

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number : 10/713,008 Confirmation No. 5744
Applicants : Masaaki IKEDA, *et al.*
Filed : November 17, 2003
Title : METHODS FOR PROLIFERATING TERMINAL
DIFFERENTIATED CELLS AND RECOMBINANT VECTORS
THEREFOR
TC/Art Unit : 1636
Examiner: : Fereydoun G. SAJJADI, Ph.D.
Docket No. : 64517.000002
Customer No. : 21967

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Sir,

I, Uichi Koshimizu, Ph.D., declare that:

1) I am a citizen of Japan residing at 15-1 Hyakuyama, Shimamoto-cho, Mishima-gun, Osaka 618-0021, Japan

2) I received a Bachelor of Sciences degree in Agriculture from the Meiji University, Kanagawa, Japan, in 1988 and a Doctor of Philosophy degree in Medicine from the Osaka University Medical School, Osaka, Japan, in 1994.

3) I am the manager and chief researcher at Asubio Pharma Co., Ltd. I have been employed by Asubio Pharma Co., Ltd. for 8 years as a researcher and have been involved in the study of regenerative medicine and gene therapy.

4) I have been associated with research in the field of cell biology, molecular biology, biochemistry, developmental and regenerative biology, gene therapy research and cardiovascular research for approximately 17 years.

5) I am an author of more than 70 publications including peer-reviewed papers, review articles, book chapters, and patent specifications. A recitation of some of these publications, together with details of my education, are given in my *curriculum vitae* which is attached as Exhibit A.

6) Based on my academic training and professional experience, I consider myself to be a person of skill in the art of regenerative biology, gene therapy research and cardiovascular research, and I was such a person prior to May 17, 2001, the foreign priority date claimed in Application No. 10/713,008 ("the '008 application").

7) I have read the '008 application, the Final Office Action mailed July 9, 2008, Applicants' response filed January 9, 2009, and the Advisory Action mailed February 9, 2009, in the '008 application.

The Claimed Invention

8) The claims relate to methods of proliferating cardiomyocytes comprising using a vector to introduce nucleotide sequences coding for a nuclear localization signal, a D-type cyclin gene, and a cyclin dependent kinase gene directly into the cardiomyocytes. Claims 31-33 and 38 recite that the vector is viral. Claim 6 recites that the vector is adenovirus.

The Enablement Rejection

9) The Examiner has rejected claims 1, 2, 4, 6, 16-33, 37, and 38 as non-enabled for the use of vectors other than adenovirus. The Examiner asserts that non-adenoviral vectors "would not impart sufficient gene expression" or that "such expression would be transient." Advisory Action, page 2. The Examiner also states that "terminally differentiated, non-replicating cells, such as cardiomyocytes, would be refractive to viral infection by retroviruses, that require actively dividing cells as hosts." *Id.* The Examiner concludes that "any vector or viral vector would not predictably provide sufficient directed delivery and expression of the cyclin and CDK genes, absent further undue experimentation." *Id.*

The Enablement Standard

10) I understand that the standard applied by the U.S. Patent and Trademark office for determining whether an invention is non-enabled is whether it would require “undue experimentation” for a person skilled in the art to make and use it. In determining whether the amount of experimentation is undue, the following factors are to be considered: (a) the breadth of the claims; (b) the nature of the invention; (c) the state of the prior art; (d) the level of one of ordinary skill; (e) the level of predictability in the art; (f) the amount of direction provided by the inventor; (g) the existence of working examples; and (h) the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

11) In making my evaluation as to whether the present invention is enabled, i.e., whether a person skilled in the art could make and use the invention without undue experimentation, I have considered the above-enumerated factors.

Numerous Non-Adenoviral Vectors Were Well Known In the Art

12) Contrary to the Examiner’s assertions, numerous methods of stably and efficiently transferring cardiomyocytes with foreign DNA were well known in the art at the time the ‘008 application was filed. Below I briefly discuss some of the literature that was widely available and that persons skilled in the art during the time the ‘008 application would no doubt have been familiar with.

13) Alexander et al. (Clin. Exp. Pharmacol. Physiol., 1999), attached as **Exhibit C**, reviews methods of gene transfer and models of gene therapy for the myocardium. Alexander states:

Gene transfer into the myocardium can be achieved through direct injection of plasmid DNA or through the delivery of viral vectors, either directly or through the coronary vasculature. Direct DNA injection has proven extremely valuable in studies aimed at characterizing the activities of promoter elements in cardiac tissue and for examining the influence of the pathophysiological state of the myocardium on expression of transferred foreign genes.

Viral vectors, in particular adenoviruses and adenoassociated virus, are capable of transfecting genetic material with high transduction efficiencies and have been applied to a range of model systems for *in vivo* gene transfer.

Efficient gene transfer has been achieved into the coronary vessels and surrounding myocardium by intracoronary infusion of adenovirus.

Because gene transfer into the myocardium can now be achieved with high efficiency in the absence of significant inflammatory responses, the ability to regulate foreign gene expression in response to an endogenous disease phenotype will enable the development of new effective viral vectors with direct clinical applicability for specified therapeutic targets.

Alexander, page 661. (emphasis added).

14) Nishizaki et al. (Ann. Thorac. Surg., 2000), attached as **Exhibit D**, teaches that gene gun-mediated transfer of an Epstein Barr virus-based episomal vector into a rat heart “results in long-lasting expression of a marker gene.” Nishizaki, page 1332. Nishizaki also teaches that “[v]arious gene transfer vectors and delivery methods have been devised to transfer genes into the heart, including direct injection of naked DNA into heart muscle, infusion of HVJ-liposomes or cationic liposomes into coronary artery, and injection of HVJ-liposomes into pericardium. *Id.* at page 1335, second column, and the references cited therein.

15) Tomiyasu et al. (Gene Therapy, 2000), attached as **Exhibit E**, teaches transduction of the left ventricular muscle of a cardiomyopathic hamster with an Epstein-Barr virus-based plasmid vector carrying human B₂-AR gene. Tomiyasu, abstract. Tomiyasu states:

Previously, we reported that a high rate of gene transduction could be achieved in cardiomyocytes *in vivo* through injection of the Epstein-Barr virus (EBV)-based plasmid. In the present study, we used this method to transduce the B₂-AR gene into the hearts of cardiomyopathic hamsters. Because direct injection of naked plasmid DNA allows transgene expression specifically in cardiac muscle, and employment of the EBV-based plasmids enables highly efficient gene transduction, our system is considered to be highly appropriate for gene therapy of heart failure.

Tomiyasu, page 2087, second column. (emphasis added). Tomiyasu also teaches that “[t]he *in vivo* genetic transduction into heart tissue has been done using many different methods, including direct intra-myocardial injection of naked plasmid DNA and adenoviral vector, and intra-coronary infusion of adenoviral, HVJ-liposome and AAV vectors.” *Id.* at 2089, second column, and the references cited therein.

16) Kaplitt et al. (Ann Thorac Surg., 1996), attached as **Exhibit F**, teaches an adeno-associated vector gene transfer into rat and porcine myocardial cells. Kaplitt, abstract. Kaplitt states that “AAV-mediated myocardial gene transfer appears to be at least as efficient as adenoviral transduction of heart cells. This is consistent with prior studies that have documented highly efficient AAV-mediated gene transfer in other organ systems.” Kaplitt, (under “Comment”) (emphasis added), and the references cited therein.

17) Debruyne et al. (Gene Therapy, 1998), attached as **Exhibit G**, teaches transfer viral IL-10 into a cardiac allograft using a liposomal vector. Debruyne states that this vector “has recently been shown to increase *in vivo* vascular gene expression by greater than 15-fold” compared with other methods of gene transfer and that this level of expression represents “a significant improvement in nonviral vector *in vivo* transfection efficiency and approach levels observed with clinically acceptable doses of adenoviral vectors.” Debruyne, page 1080. (emphasis added). Debruyne also states that “[p]revious studies from our laboratory showed that a variety of plasmid and viral (retrovirus, herpesvirus, adenovirus) vectors can successfully transfer and express exogenous genes in cardiac allografts.” *Id.* (emphasis added), and the references cited therein.

18) Kawaguchi et al. (Circulation, 1997), attached as **Exhibit H**, teaches successful transfection of the human eNOS gene into rat hearts using Sendai virus-coated liposomes. Kawaguchi, abstract. Kawaguchi concludes that the “transfection efficiency was comparable to adenovirus-mediated transfection” and “caused no inflammation.” *Id.* (emphasis added).

19) Trivedi et al. (J. Neurochemistry, 1995), attached as **Exhibit I**, teaches that cationic liposomes can be used to deliver recombinant genes to myoblasts at high efficiency. Trivedi, abstract.

20) Losordo et al. (Circulation, 1998), attached as **Exhibit J**, describes a phase 1 clinical trial to determine the safety and bioactivity of direct myocardial gene transfer of VEGF as a therapy for patients with symptomatic myocardial ischemia. Losordo states that “[t]his initial experience with naked gene transfer as sole therapy for myocardial ischemia suggests that direct myocardial injection of naked plasmid DNA, via a minimally invasive

chest wall incision, is safe and may lead to reduced symptoms and improved myocardial perfusion in selected patients with chronic myocardial ischemia.” Losordo, abstract.

21) Tio et al. (Human Gene Therapy, 1999), attached as **Exhibit K**, teaches that direct intramyocardial injection of naked DNA encoding VEGF is safe and can enhance collateral development and improve myocardial perfusion. *See* Tio, abstract.

22) Lin et al. (Circulation, 1990), attached as **Exhibit L**, teaches that plasmid DNA can be introduced into and expressed in adult rat cardiac myocytes for at least four weeks. Lin, page 2217, second column.

23) Buttrick et al. (Circ. Res., 1992) and Kitsis et al. (PNAS, 1991), attached as **Exhibits M** and **N**, respectively, also teach that plasmid DNA can be introduced into and expressed in adult rat hearts.

24) Sakoda et al. (J. Mol. Cell Cardiol., 1999), attached as **Exhibit O**, teaches “the efficient transduction of non-dividing cells, including post mitotic beating rat cardiac myocytes and well differentiated rat L6 myofibers” using the retrovirus *Lentivirus* as a vector. Sakoda, abstract. (emphasis added).

25) Mochizuki et al. (J. of Virology, 1998), attached as **Exhibit P**, teaches successful transduction of rat cardiac myocytes using an HIV-1 retroviral vector system. Mochizuki concludes that this vector system is “efficient, robust, and safe.” Mochizuki, page 8882. (emphasis added).

Conclusion

26) As the references summarized above amply demonstrate, there were numerous well known non-adenoviral methods and vectors for stably and efficiently introducing foreign genes into cardiomyocytes prior to May 2001. Included among these are direct injection of plasmid DNA, gene-gun mediated transfer of plasmids and viral-based vectors, liposomal transfer of vector DNA, transfection with adeno-associated vector DNA, and transduction with retroviral-based vectors. These references directly refute the Examiner’s contention that, prior to May 2001, non-adenoviral and retroviral vectors were incapable of stably and efficiently transforming cardiomyocytes with foreign DNA.

I declare that all statements made herein are based on personal knowledge or upon information and belief and are believed to be true; and further that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Dated: May 2, 2009



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EXHIBIT A

Exhibit A

Curriculum Vitae

June 12, 2007

Name: Uichi KOSHIMIZU

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**15-1 Hyakuyama, Shimamoto-cho, Mishima-gun,
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Position: Manager and chief researcher of Medical Biotechnology Group

Date of Birth: June 21, 1965 (Age:41)

Nationality: Japanese

Education and Positions:

**1984-1988 Faculty of Agriculture at Meiji University (Knagawa, Japan)
Advisor: Prof. H. Nagasawa**

**1988-1994 Ph.D. candidate of Osaka University Medical School (Osaka,
Japan)**

Advisor: Prof. Y. Nishimune

(Research Insitute for Microbial Discases)

**1993-1995 Research fellow, National Institute of Genetics (Mishima,
Japan)**

Advisor: Prof. N. Nakatsuji

**Received Ph.D. in March, 1994, from Osaka University Medical
School**

1995-2001 **Assistant Professor & Lecturer, Osaka University Medical School (Osaka, Japan)**

2001-2007 **Senior Researcher,
Suntory Biomedical Research Co. Ltd.
(renamed as Asubio Pharma Co. Ltd)**

2007- **Manager and chief researcher of Medical Biotechnology Group**

Awards: **Fellowships of the Japan Society for the Promotion of Science
for Junior Scientists (1992-1994, 1994- 1995)**

Memberships: **Japanese Society of Developmental Biologists
Molecular Biological Society of Japan
Japanese Society of Regenerative Medicine
Japan Society of Gene Therapy
International Society for Stem Cell Research**

Publication List

Articles (in English): 45

Reviews (in English): 1

Reviews (in Japanese): 25

Patent specifications: 4

The important articles

1. U. Koshimizu, D. Watanabe, Y. Tajima, and Y. Nishimune
Effects of *W* (*c-kit*) Gene Mutation on Gametogenesis in Male Mice: Agametic Tubular Segments in *W^h/W^h* Mice. *Development* 114:861-867 (1992)
2. T. Tono, T. Tsujimura, U. Koshimizu, T. Kasugai, S. Adachi, K. Isozaki, S.-I. Nishikawa, M. Morimoto, Y. Nishimune, and Y. Kitamura
c-kit Gene Was Not Transcribed in Cultured Mast Cells of Mast Cell- Deficient *W^h/W^h* Mice That Have a Normal Number of Erythrocytes and a Normal *c-kit* Coding Region. *Blood* 80:1448-1453 (1992)
3. U. Koshimizu, D. Watanabe, Y. Tajima, and Y. Nishimune
A Novel Stage-Specific Differentiation Antigen Is Expressed on Mouse Testicular Germ Cells during Early Meiotic Prophase. *Biol. Reprod.* 49:875-884 (1993)
4. T. Tsujimura, U. Koshimizu, H. Katoh, K. Isozaki, Y. Kanakura, T. Tono, S. Adachi, T. Kasugai, H. Tei, Y. Nishimune, S. Nomura, and Y. Kitamura
Mast Cell Number in the Skin of Heterozygotes Reflects the Molecular Nature of *c-kit* Mutation. *Blood* 81:2530-2538 (1993)
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6. D. Watanabe, K. Yamada, Y. Nishina, Y. Tajima, U. Koshimizu, A. Nagata, and Y. Nishimune
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8. U. Koshimizu, H. Nishioka, D. Watanabe, K. Dohmae and Y. Nishimune
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9. U. Koshimizu, M. Watanabe and N. Nakatsuji
Retinoic Acid Is a Potent Growth Activator of Mouse Primordial Germ Cells *in vitro*.
Dev. Biol. 168: 683-685 (1995)
10. U. Koshimizu, T. Taga, M. Watanabe, M. Saito, Y. Shirayoshi, T. Kishimoto and N. Nakatsuji
Functional Requirement of gp130-mediated Signaling for Growth and Survival of Mouse Primordial Germ Cells *in vitro* and Derivation of Embryonic Germ (EG) Cells.
Development 122:1235-1242 (1996)
11. U. Koshimizu, H. Takahashi, Y. Yoshida and T. Nakamura
cDNA Cloning, Genomic Organization, and Chromosomal Localization of Mouse LIM Motif-Containing Kinase *Link-2*.
Biochem. Biophys. Res. Commun. 241:243-250 (1997)
12. U. Koshimizu, K. Matsumoto, T. Nakamura
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Expression of a Novel Type of Classic Cadherin, PB-Cadherin in Developing Brain and Limb Buds. *Dev. Dyn.* 215:206-214 (1999)
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Arch. Oral Biol. 44:935-46 (1999)
17. T. Kunisada, H. Yamazaki, T. Hirobe, S. Kamei, M. Omoteno, H. Tagaya, U. Koshimizu, T. Nakamura, and S. Hayashi
Keratinocyte Expression of Transgenic Hepatocyte Growth Factor (HGF) Affects Melanocyte Development, Leading to Dermal Melanocytosis.
Mech. Dev. 94:67-78 (2000)
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Functional Involvement of *Xenopus* LIM-Kinases in Progression of Oocyte Maturation.
Dev Biol. 229:554-67 (2001)

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Hepatocyte Growth Factor Is Essential for Migration of Myogenic Cells and Promotes Their Proliferation during the Early Periods of Tongue Morphogenesis in Mouse Embryos.
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Molecular Cloning of AMSH-Like Molecule and Its Unique Expression in Testis.
Biochem. Biophys. Acta (2003)

22. S. Yuasa, Y. Hatahshi, U. Koshimizu, T. Tanaka, K. Sugimura, M. Kinoshita, F. Hattori, S. Fukami, T. Shimazaki, S. Ogawa, H. Okano, and K. Fukuda.
Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells.
Nat. Biotechnol. 23:607-11(2005)

23. M. Nagaoka, U. Koshimizu, S. Yuasa, F. Hattori, H. Chen, T. Tanaka, M. Okabe, K. Fukuda, and T. Akaike
E-cadherin-coated plates induce rapid proliferation, efficient transfection, and low LIF dependency of pluripotent ES cells without colony formation..
PLoS ONE. 1: e15 (2006)

24. M. Nagaoka, H. Ise, I. Harada, U. Koshimizu, A. Maruyama, and T. Akaike
Embryonic undifferentiated cells show the scattering activity on E-cadherin-immobilized surface. *J. Cell. Biochem.* (in press)

25. M. Tamamori-Adachi, H. Takagi, K. Hashimoto, K. Goto, T. Hidaka, U. Koshimizu, K. Yamada, I. Goto, J. Kawauchi, K. I. Nakayama, N. Inomata, and S. Kitajima
in situ proliferation of cardiomyocytes by nuclear cyclin D1/CDK4 and Skp2 improves heart failure.(submitted)

26. T. Tanaka, M. Kadokura, K. Kawashima, S. Oikawa, K. Fukuda, and U. Koshimizu
Transient activation of canonical Wnt signaling induces cardiomyocyte differentiation of embryonic stem cells. (in preparation)

Patent specifications

1. "Method of inducing the differentiation of stem cells into myocardial cells"
(WO2005-033298)
2. "Method of growing myocardial cells" (WO2005-049822)
3. "Method of proliferating pluripotent cells" (WO2005-090557)
4. "Method of preparation of cardiomyocytes from pluripotent stem cells"
(PCT/JP2007/59242)

EXHIBIT C

BRIEF REVIEW

GENE TRANSFER AND MODELS OF GENE THERAPY FOR THE MYOCARDIUM

M Yvonne Alexander,[†] Keith A Webster,[‡] Patricia H McDonald^{*†} and Howard M Prentice^{*}

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SUMMARY

1. Gene transfer into the myocardium can be achieved through direct injection of plasmid DNA or through the delivery of viral vectors, either directly or through the coronary vasculature. Direct DNA injection has proven extremely valuable in studies aimed at characterizing the activities of promoter elements in cardiac tissue and for examining the influence of the pathophysiological state of the myocardium on expression of transferred foreign genes.

2. Viral vectors, in particular adenoviruses and adeno-associated virus, are capable of transfecting genetic material with high transduction efficiencies and have been applied to a range of model systems for *in vivo* gene transfer. Efficient gene transfer has been achieved into the coronary vessels and surrounding myocardium by intracoronary infusion of adenovirus.

3. Because the immunogenicity of viral vectors can limit transgene expression, much attention has been paid to strategies for circumventing this, including the development of new modified adenovirus and adeno-associated virus vectors that do not elicit significant inflammatory responses. While cellular transplantation may prove valuable for the repair of myocardial tissue, confirmation of its value awaits establishment of a functional improvement in the myocardium following cell grafting.

4. Because gene transfer into the myocardium can now be achieved with high efficiency in the absence of significant inflammatory responses, the ability to regulate foreign gene expression in response to an endogenous disease phenotype will enable the development of new effective viral vectors with direct clinical applicability for specified therapeutic targets.

Key words: adenovirus, animal models of foreign gene delivery, gene therapy, gene transfer, myocardial ischaemia, myocardium, viral vectors.

INTRODUCTION

Analyses of gene expression and promoter function in the intact heart are now possible through the advent of direct injection techniques to efficiently deliver plasmid and viral DNA into the myocardium. These techniques not only allow molecular genetic analyses of gene expression under different developmental and diseased states, but have also opened up new possibilities for gene therapy. Gene transfer into the heart and coronary vasculature by direct injection of naked, emulsified or encapsulated DNA is likely to have clinical as well as research applications in the very near future.

Injection of plasmid DNA, naked or combined with various mixtures of cationic lipids, has resulted in detectable levels of foreign gene expression *in vivo* in both skeletal and cardiac muscle. Viral vectors, in particular adenovirus (Ad) and adeno-associated virus (AAV), transfect genetic material with even higher transduction efficiencies and there is some debate about which transfer system is the best.^{1–4} We will give an account of the recent advances of gene transfer using the different viral and non-viral methods, illustrating the important features of each approach. The strengths and weaknesses of the current vector systems will be reviewed and we will examine how vectors could be optimized for long-term gene expression. Finally, we will examine the potential for efficient delivery of these vectors in the clinical setting by intravenous injection and catheter-based techniques.

GENE TRANSFER IN THE MYOCARDIUM: DIRECT DNA INJECTION

Studies on the regulation of gene expression in the myocardium have relied heavily on transfection into primary cells, in particular neonatal cardiac myocytes and, to a more limited extent, adult myocytes. There are still no well-defined cardiac cell lines, although Simian virus 40 (SV40) large T-transformed atrial cells can be passaged and retain a cardiac phenotype.⁵ Adult myocytes are difficult to isolate and maintain in culture for gene expression studies; they are also difficult to transfect and tend to dedifferentiate in long-term culture. While a wealth of information has been obtained using rat neonatal cardiac myocytes, these systems have been criticized for being *in vitro* as well as neonatal and rodent and, therefore, not an appropriate model for the analysis of heart function, neither diseased nor developmental. Although the neonatal rat cardiac myocyte

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List of abbreviations:

AAV	Adeno-associated virus	Luc	Luciferase
Ad	Adenovirus	α -MHC	α -Myosin heavy chain
β -Gal	Beta galactosidase	MuLV	Murine leukaemia virus
cTnC	Cardiac troponin C	p.f.u.	Plaque-forming unit
HRE	Hypoxia response element	PTGT	Percutaneous transluminal gene transfer
HSP	Heat-shock protein	SV40	Simian virus 40
HSV	Herpes simplex virus	Tet	Tetracycline
HVJ	Haemagglutinin virus of Japan	TGF- β	Transforming growth factor- β .
kb	Kilobase	VEGF	Vascular endothelial growth factor
LTR	Long-terminal repeat.		

models have generated important information on the regulation of gene expression in cardiac cells, as well as implications for disease responses, these results need to be validated in different species and in the context of the whole intact heart (*in vivo*). Cultures of human cardiac myocytes are likely to be important in this respect⁶ and the recent explosion in transgenic mouse studies has certainly featured prominently and is likely to continue to do so in the future. In our opinion, combinations of the three systems (virus delivery *in vitro*, transgenic and direct injection) will most efficiently generate a complete molecular and cellular picture of heart disease and development. Direct transfer of DNA into cardiac muscle or vasculature currently has advantages over transgenic experiments in being cheaper, applicable to large as well as small animals and applicable to a wide range of developmental as well as disease models. Also, direct transfer of DNA into the adult cardiovascular system is likely to precede germ-line manipulations in the context of gene therapy.

In vivo transfection has been especially attractive for the myocardium, in terms of the information that could be determined about promoter activation in the whole heart responding to physiological (and pathophysiological) function under the influences of naturally occurring mechanical and hormonal stimuli. It was first shown by Wolff *et al.*⁷ that injection of naked plasmid DNA directly into skeletal muscle of mice permitted reproducible expression and high fidelity of the foreign gene's promoter function. Subsequently, it was demonstrated that promoter chimeras injected into the heart responded appropriately to physiological hormonal stimuli⁸ and, furthermore, that *cis*-acting elements may act differently *in vivo* compared with cell culture models.⁹ The direct injection methodology combines some of the advantages of the *in vitro* transfection approach and of the transgenic mouse models in its application to short-term analysis of gene regulation.

Direct DNA injection has been especially valuable for studies directed at characterizing the activities of promoter elements in cardiac tissue and for examining transcriptional responses to physiological stimuli.¹⁰ It was first demonstrated by Kitsis *et al.* that promoter elements transfected into the heart by direct DNA injection were capable of tissue-specific regulation and could respond appropriately to alterations in thyroid hormone levels *in vivo*.⁸ The technique of direct DNA injection has also been applied to the characterization of the heart-specific activation properties of a 156 b.p. promoter region located immediately 5' to the transcriptional start site of the slow/cardiac troponin C (*cTnC*) gene.¹¹ Other cardiac-specific promoter domains for the gene for the M isozyme of creatine kinase and for a ventricular-specific myosin light chain have been similarly characterized by direct injection of plasmid constructs. The method of direct injection may also be useful for

investigating the effects of specific pathophysiological states on cardiac gene expression, such as ischaemia, hypertension and pressure or volume overload.^{10,12,13}

Many groups have demonstrated the expression of naked plasmid DNA in the rat heart.^{8,12,14–16} Our laboratory was the first to show that the pathophysiological state of the myocardium *in vivo* could dramatically influence the expression of transferred foreign genes^{10,17} (and *vide infra*). There have been some discrepancies concerning reported stability of plasmid-based transgenes in both skeletal and cardiac muscle, but it seems likely that long-term expression of 3–4 months is quite feasible.¹⁸ A gradual decline of transgene expression may be expected due to the episomal localization of the DNA in post-mitotic cardiac and skeletal muscle cells.^{14,18}

The capacity to take up and express plasmid DNA following direct injection appears to be quite unique for striated muscle and does not occur in other organs at significant levels. The reasons for this are not clear, but it has been suggested that the cell damage and inflammation caused by the injection needle could mediate gene transfer by causing membrane disruption.¹⁹ From electron microscope studies on injected skeletal muscle, Wolff *et al.*²⁰ demonstrated that colloidal gold, conjugated to plasmid DNA, crossed the external lamina and entered T tubules and caveolae, while gold conjugated with polylysine, polyethylene glycol or polyglutamate remained outside the fibres. These data suggest that DNA entry does not occur through transient membrane disruptions and does not appear to result from endocytosis. Wolff *et al.* suggest an alternative mechanism for DNA uptake by some type of cell membrane transporter, in particular via photocytosis. Microinjection of plasmid DNA into the cytoplasm of primary rat myotubes resulted in DNA entry into post-mitotic nuclei through the nuclear pore by a process common to other large karyophilic macromolecules.²¹ This understanding of plasmid DNA nuclear entry may provide a basis for increasing the efficiency of direct plasmid gene transfer into heart and skeletal muscle. Directly injected plasmid DNA results in higher transfection efficiencies in the myocardium than in skeletal muscle when both tissues are injected with identical plasmid DNA in the same manner.⁸ While the reason for this is unclear, it is possible that differences in structure of the T tubule system between skeletal and cardiac muscle may contribute to the discrepancies in transfection efficiency.²⁰

Direct injection of naked plasmid DNA into the myocardium has only limited potential as a method for cardiac gene therapy because of the small number of cells per injection that express the transgene. Expression, as determined by histochemistry, appears to be localized around the site of injection with as few as 100–200 cells expressing the gene product.¹⁴ Several investigators have studied the

parameters affecting gene expression following direct DNA injection. Increasing the quantity of DNA used for myocardial transfection did not alter levels of foreign gene expression,²² whereas increasing the volume of injectate, while maintaining a constant quantity of DNA (50 µg in this case) appeared to increase expression from a luciferase-encoding plasmid. It has been further demonstrated that transfection efficiencies are higher with closed circularized DNA than with linearized DNA,¹⁴ but the total number of expressing cells remains small and the technique is probably only suitable for secreted gene products.

A critical requirement for gene therapy protocols is the ability to achieve control of the foreign gene expression appropriately in the host. Fishman *et al.*²³ have developed a tetracycline (tet)-regulated gene expression system, based on injection into adult rat hearts of a Tet-repressor VP16 transactivator plasmid with a luciferase (luc) target gene. Using this system it was possible to induce luciferase expression by two orders of magnitude in response to small changes in input-controlled transactivator DNA, allowing target gene expression to be induced or repressed by altered antibiotic administrations.

Our own studies have demonstrated that specific promoter elements can be used to regulate transgene expression in response to physiological stress.^{10,17} In our experiments, we examined the degree of regulation of hypoxia-responsive promoter constructs in an experimental model of myocardial ischaemia with reperfusion. Hypoxia is a critical and obligatory component of ischaemic tissue and solid tumours, where the hypoxic zone is restricted to the diseased tissue. It should be possible to tightly regulate transgene expression in these diseased tissues through the use of hypoxia as an endogenous regulator. To investigate transgene regulation in myocardial ischaemia, we constructed an expression plasmid containing multiple copies of a hypoxia response element (HRE) from the erythropoietin gene placed upstream of a minimal α -myosin heavy chain (α -MHC) promoter driving a luciferase reporter. In cell culture, expression of this test promoter (α -MHC86HRE) was highly muscle specific and was induced approximately 10-fold by hypoxia. In a rabbit model of 15 min myocardial ischaemia followed by reperfusion, expression of the construct was induced by four- to five-fold at 1 and 4 h post-ischaemia, returning to basal levels by 8 h.¹⁰ These results show, for the first time, that it is possible to tightly regulate a transgene plasmid or virus vector in response to the disease phenotype and this represents a significant milestone in gene therapy research.

VECTORS FOR MYOCARDIAL GENE TRANSFER

Retroviruses

The retrovirus is a small RNA virus, packaged within a glycoprotein envelope. The life cycle of the retrovirus is now well characterized.²⁴ Following infection into its host, the viral RNA is reverse-transcribed into a double-stranded DNA molecule that then becomes integrated into the cellular genome. By exploitation of this stable integration into the host genome following infection, it was anticipated that long-term expression of the recombinant gene would be achieved. However, as an unexplained loss of exogenous gene expression is frequent with retroviral vectors and integration of retroviral sequences is dependent on cell division,²⁵ they may prove to be of little use for the myocardium. Nevertheless, initial experiments with

retroviral vectors carrying the β -galactosidase (β -gal) gene showed promising results using *ex vivo* methods in heart and skeletal muscle; Salvatori *et al.* achieved 50% gene transfer efficiency in foetal myoblasts and adult satellite cells, which in turn led to expression in muscle fibres following *in vivo* transplantation.²⁶ A similar retrovirus carrying the β -gal reporter gene was used for infection of murine foetal cardiac myocytes in culture, which were then transplanted into syngeneic adult mice.²⁷ Histological analysis of the grafted hearts indicated expression of the β -gal gene product in the transplanted cells for as long as 6 months and revealed an absence of inflammation or scar tissue over this time period.²⁷ Clinical use of retroviruses as gene therapy vehicles may be limited by the potential for oncogenicity and insertional mutagenesis. Retroviral protocols are also limited by the relatively low viral titres that can be obtained and by the limited size of foreign sequence they can accommodate.²⁸

Adenoviruses

Replication-deficient adenovirus (Ad) are currently among the most efficient vectors for transferring genes to a wide variety of cell types *in vivo*.^{2,3} In a comparison of gene transfer using plasmid DNA and recombinant Ad DNA, it was found that the efficiency of transduction with Ad was at least an order of magnitude higher than that observed with plasmid DNA, tissue penetration was vastly improved and transgene expression was proportionately increased.²⁹ Unique properties of adenoviral vectors include an exceptionally high efficiency of infection and the ability to accommodate large fragments of foreign DNA (up to 8 kb). Of particular importance for gene transfer to the myocardium is the property to efficiently infect both proliferating and terminally differentiated cells. In contrast with retroviruses, Ad can be prepared at much higher stock concentrations than retroviruses, with typical titres of 10^{11} plaque-forming units (p.f.u.)/mL, 10 000-fold higher than equivalent retroviral titres. Furthermore, the adenoviral genome remains episomal, so that the potential for oncogenesis and insertional mutagenesis is avoided.

As with all current vectors, there are limitations to the use of the Ad. The first-generation of Ad5 vectors has most of the Ad genome and expresses multiple Ad proteins, including the penton protein, which is associated with cytotoxicity, particularly at a high multiplicity of infection.³⁰ Virus capsid proteins also stimulate a humoral response, activating neutralizing antibodies, inflammation and elimination of both Ad vector and host cell.³¹ Second- and third-generation Ad vectors, in which a region of 28 kb spanning all adenoviral coding sequences has been deleted, have recently been developed to circumvent the inflammatory responses associated with Ad infection. Such vectors, which require helper viruses for their generation, can accommodate large DNA fragments encoding multiple foreign genes.³²

Adeno-associated viral vectors

Because of the inflammatory responses associated with infection by Ad and other viral vectors, attention has been paid to the potential for using viruses that are defective in that they express no viral genes. Adeno-associated virus is an example of such a defective virus, devoid of viral genes but capable of permitting foreign genes to be packaged into a viral coat.^{4,33} This non-pathogenic human parvovirus differs from Ad in that it generally integrates into a specific site in the genome of the host cell and may provide for longer-term

transgene expression, although producing sufficiently high titres remains a problem.^{1,34} Direct injection of AAV into rat hearts resulted in transgene expression 2 months after administration of the virus, with no detectable inflammatory response.³⁵ Delivery of an AAV vector into the coronary vasculature of pigs by percutaneous intra-arterial infusion using a routine catheter technique resulted in expression in cardiac myocytes for at least 6 months after injection, without toxicity or inflammation.^{33,35–43} Because of its high infectivity and low immunogenicity, AAV gene transfer shows potential as a highly effective gene transfer vector for myocardium, in particular as methods become available for increasing the yield of virus⁴⁴ and for increasing transduction efficiencies by infecting in the presence of Ad gene products.⁴²

Herpes simplex virus

The ability of Herpes simplex virus (HSV1) vectors to accommodate very large foreign DNA fragments has rendered them attractive as a gene transfer system with potential advantages over other viral systems. While HSV1 has been proposed as a candidate gene therapy vector for the nervous system because of its ability to latently infect neurons, it is also capable of infecting a range of other cell types where the vector may prove valuable if lytic infection can be inhibited. Coffin *et al.* achieved successful gene transfer in cardiac myocytes and vascular smooth muscle cells *in vitro* using three different disabled HSV vectors, with an efficiency of transfection of the vascular smooth muscle cells that was less than that observed with myocytes. In the rat heart *in vivo*, successful transfection was obtained with no apparent change in cellular morphology, suggesting negligible cytopathic effects. Mesri *et al.* used a replication-defective HSV1 vector carrying the gene encoding the angiogenic factor vascular endothelial growth factor (VEGF) to infect fibroblasts *in vitro* for subsequent transplantation studies. When the infected fibroblasts were injected into syngeneic mice, transgene expression was found to be capable of eliciting an angiogenic response.⁴⁵ This study pointed to the possibility that a HSV1 vector could have potential for applications for inducing focal angiogenesis to ameliorate myocardial ischaemia. Because HSV1 can transfer large DNA fragments, such gene therapy vectors may be particularly applicable to myocardial disorders requiring large genomic substitutions or the use of extensive genetic control regions.

Haemagglutinin virus of Japan

The haemagglutinin virus of Japan (HVJ) is an inactivated paramyxovirus that has been used within a liposome complex that entraps DNA. In transfections of cardiac myocytes in culture, it was shown that 90% transfection efficiency could be obtained and that foreign gene expression was detectable over 7 days.⁴⁶ Intracoronary injection of the HVJ/liposome complexes was also effective for providing efficient gene transfer into cardiac myocytes *in vivo*, with expression lasting for 1 week.⁴⁷ Directly injected HVJ/liposome DNA has been shown to result in higher reporter gene expression 3 days after administration than that obtained from injection of naked plasmid DNA.⁴⁶ Introduction of the HVJ/liposome complex containing a β -gal-encoding vector within the pericardium resulted in widespread staining of cardiac myocytes and fibroblasts. The HVJ-mediated gene transfer by direct infusion into the coronary artery resulted in staining of cardiac myocytes around the microvasculature.⁴⁶ *In vivo* intracoronary infusion of the HVJ/liposome complex containing the heat-shock protein 70 (HSP70) gene in rats resulted in enhanced

tolerance of Langendorff perfused hearts to ischaemia–reperfusion injury.⁴⁸

MODEL SYSTEMS FOR *IN VIVO* GENE TRANSFER

In vivo studies using small animal models

Several groups have assessed the specificity, efficiency and duration of Ad-mediated gene expression in the myocardium.^{22,49–54} Stratford-Perricaudet *et al.*⁴⁹ were the first group to demonstrate long-term *in vivo* gene transfer throughout mouse skeletal and cardiac muscles after intravenous administration of a recombinant adenovirus (1×10^9 p.f.u./mL). It was demonstrated that approximately 0.2% of neonatal cardiomyocytes were transduced at 15 days after injection of the Ad. Reporter gene expression was found to persist, but decreased over a period of 12 months. In adult mice, intravenous Ad administration resulted in less efficient gene transfer.⁴⁹

Guzman *et al.* used a sub-diaphragmatic approach for myocardial gene transfer⁵⁰ rather than the thoracic approach of previous studies^{8,18} in order to avoid the high mortality associated with intubation and ventilation. In an analysis of myocardial gene transfer in the rat by sub-diaphragmatic injection of 5×10^8 p.f.u. Ad or 200 μ g plasmid DNA,⁵⁰ it was demonstrated that Ad delivery was considerably more efficient than plasmid injection. Adenoviral injection resulted in significant foreign gene expression in cardiac myocytes, with maximal expression during the first week following injection, decreased transgene expression at 10–15 days after injection and no detectable foreign gene expression at 30 days. These investigators observed an acute inflammatory response in hearts injected with Ad. Because an inflammatory response has also been noted with plasmid DNA injection,^{18,22} the authors suggest the inflammatory response may be related to injury produced by direct injection rather than to a stimulation of an immunological response to viral gene products.

In vivo studies using large animals: Relevance to humans

While many cardiac studies have been carried out in small animal models that may be informative in terms of cardiac function and ventricular performance under various inotropic and loading conditions,⁵⁵ it is important to use large animal models with physiology similar to that of a human and for consideration of potential applications to human gene therapy protocols. In a pig model, injection of replication-deficient recombinant Ad vectors carrying the *Luc* gene resulted in significant reporter gene activity detected at 3 days, increasing markedly at 7 days and then declining progressively at 14 and 21 days.⁵⁶ Comparable levels of foreign gene expression were found between plasmid and Ad injection but, when compared on a molar basis (i.e. when normalized to the number of genes injected), Ad-mediated gene transfer was found to be 140 000-fold more efficient than plasmid delivery. Injection of a β -gal-encoding Ad resulted in foreign gene expression that was localized predominantly to cardiac myocytes. The amount of recombinant protein expressed correlated closely with the quantity of virus injected in a 100 μ L volume, over a range of virus concentrations ranging from 0.7×10^9 to 3.6×10^9 p.f.u./mL. No detrimental effects on ventricular function were found, although there was evidence of pronounced leucocytic infiltration in the virally infected myocardium.⁵⁶ In trying to circumvent the immune response, Kaplitt *et al.*³⁵ delivered an AAV

vector percutaneously by intra-arterial infusion into the coronary vasculature of a pig. Delivery of this vector by a routine catheter technique resulted in transgene expression in cardiac myocytes for at least 6 months after injection without toxicity or inflammation.³⁵

DELIVERY METHODS

One of the key elements in successful gene transduction of the target cells is the method used in delivering the viral vector or plasmid DNA to the host cells. Initial attempts to programme recombinant gene expression in the myocardium were limited by low efficiencies of gene transfer and by the need for intramyocardial injections of DNA or Ad.^{14,50,53} Barr *et al.*⁵⁷ described a more efficient method for gene transfer into both the coronary vessels and surrounding myocardium, using catheter-mediated gene transfer. Intracoronary infusions of Ad are relatively non-invasive and can be performed percutaneously using established cardiac catheterization techniques. Unlike direct injection into the myocardium, intracoronary infusion of Ad into the rabbit myocardium did not induce inflammation or myocardial necrosis.⁵⁷ As many as 32% of cardiac myocytes expressed the recombinant gene at 5 days following infusion, but only 0.01% of cells in four of seven animals expressed the gene at 1–2 months. However, levels of gene expression in the myocardium were 10–50-fold higher by comparison with those obtained by direct DNA injection. In a similar study, Li *et al.* examined the feasibility, efficiency and safety of Ad-mediated gene transfer *in vivo* into canine myocardium by percutaneous transluminal gene transfer (PTGT) using a needle catheter.⁵⁸ Injections into the left ventricle of dogs through a needle catheter inserted via a femoral artery were performed using either replication-deficient Ad or plasmid, both expressing β -gal. Expression of lacZ was examined by histochemical staining and quantified by measuring β -gal activity. Injection with 1.0×10^9 p.f.u. recombinant Ad induced lacZ expression at levels at least 10-fold higher than those obtained with a 50 μ g injection of plasmid-expressing lacZ. Foreign β -gal expression was detected within 24 h, peaked at 7 days and persisted for 2 weeks after gene transfer. The duration and levels of gene expression coincide with the results reported by Barr *et al.*⁵⁷ A comparison of delivery methods in porcine heart indicated that intramyocardial injection of recombinant Ad was more efficient for short-term gene transfer than intracoronary infusion.⁵³ This observation was in contrast with data from intracoronary infusion of AAV using these standard catheterization techniques and demonstrating successful long-term gene transfer in the porcine model.³⁵ As an alternative to intravascular administration, Lamping *et al.*⁵⁹ have used an Ad encoding a nuclear-targeted β -gal to investigate vector delivery into the pericardial sac of dogs. One day after injection, transgene expression was observed in the parietal pericardium and left atrial tissue, with lower levels detectable in the right and left ventricles. Histochemical analysis indicated expression of the transgene in the visceral pericardium of atria and ventricles and, occasionally, in the epicardial myocytes, arterioles and venules.⁵⁹

IMMUNOGENICITY AND NEW VECTOR DESIGN

Because a major barrier in obtaining efficient gene transfer has been the problem of transient foreign gene expression and an inflammatory response, it has become necessary to focus on the nature of the immune response and to consider ways of circumventing it. Studies

aimed at identifying the immune effector involved in determining the longevity of virally delivered transgene expression have demonstrated that immunodeficient rats display higher levels and more prolonged durations of foreign gene expression than immunocompetent rats.⁶⁰ Ablation of CD4+ T cell activation at the time of vector administration has been used as a strategy for preventing cellular and humoral immunity.⁶¹ By administration of immune-modulating agents, such as monoclonal antibodies to CD4+ cells and to CD40 ligand, it has been possible to prevent the effector response of CD8+ T and B cells.⁶¹ A comparable immunosuppressive effect has been reported in studies using transforming growth factor (TGF)- β 1 or *vil10*.⁴⁰

An alternative strategy to immunomodulation is the development of novel viral vectors that may elicit decreased immune responses. Heavily deleted gutless Ad vectors that are depleted of all viral genes are capable of encoding multiple foreign genes and can retain their ability to infect target cell types.^{62–64} An Ad dodecahedron made of Ad pentons or penton bases retains many of the properties of Ad, including efficiency of entry and efficient release of DNA from endosomes, but may have the advantage of being non-immunogenic.⁶⁴ These gutless Ad vectors and the AAV vectors currently hold the most promise for the future as vectors for delivery of therapeutic genes to the heart and vasculature.

CELLULAR TRANSPLANTATION

Myocardial regeneration and autologous grafting

Because the adult mammalian myocardium is incapable of regeneration, considerable attention has been directed towards replacing damaged myocardium with new viable muscle.⁶⁵ Cardiac myocytes transformed with SV40 large T antigen, which have been used in pilot experiments for grafting into hearts of syngeneic mice, were shown to fuse successfully with the host myocardium and were viable for as long as 4 months postimplantation.⁶⁶ The transformed nature of the transplanted cells from these studies led to concerns over their unregulated growth potential. The grafts were not viable in the long term and there was no evidence for improved performance of the myocardium.

In studies on the transplantation of skeletal myoblasts into ventricular myocardium, C2C12 skeletal myoblasts were shown to be capable of forming long-term differentiated grafts with evidence of intercalated discs and gap junctions with aligned cardiac cells and repressed myoblast proliferation in the hearts of syngeneic mice.⁶⁷ Again, there was evidence of tumorigenesis and no evidence for improved cardiac function. Allogeneic and xenogeneic myoblasts injected into the anterior and posterior walls of the porcine left ventricle were successfully transplanted in immunosuppressed animals with no significant graft rejection.⁶⁸ No reports have yet claimed to show changes in myocardial function in response to cellular transplantation.

Foetal cardiac myocytes have been successfully implanted into the hearts of adult mice with a demonstration of the presence of intercalated discs between the grafted cells and the host myocardium.⁶⁹ Following engraftment of foetal cardiomyocytes into the myocardium of dystrophic mice and dogs, there was clear morphological evidence of spontaneous fusion of host and donor cells.⁷⁰ Autologous transplantation of cardiac myocytes would be clearly preferable to avoid graft rejection, but the accessibility of donor cardiac myoblasts is likely to limit the clinical applicability unless there are breakthroughs in regeneration research. Cardiac myocytes differentiated

in culture from embryonic stem cells⁷¹ may eventually provide a source of cells/tissue for *in vivo* grafting experiments.⁴⁰

One means of achieving immunosuppression as well as delivering therapeutic genes has been to inject genetically engineered skeletal myoblasts into the myocardium or skeletal muscle where they secrete a foreign gene product.^{72,73} Retrovirally transduced allografts, expressing TGF- β 1 under the control of the SV40 promoter or interleukin (IL)-10 gene under the control of murine leukaemia virus long-term repeat (MuLV)-LTR, displayed increased survival consistent with an induced immunosuppression.⁷²

Cellular transplantation into scar or infarct tissue

An alternative approach for myocardial repair may be to induce differentiation of skeletal muscle within cardiac muscle from transplanted satellite cells. Skeletal muscle satellite cells are undifferentiated myoblasts that remain dormant under the basal lamina but become activated upon myofibre injury, when they migrate towards the damaged site, enter a mitotic cycle and differentiate into skeletal muscle.⁷⁴ Satellite cells, when implanted into injured myocardium, can be influenced by the myocardial environment to display a cardiac myofibre-like phenotype.^{75,76} Such cells implanted into a site of cryoinjury were found to differentiate into muscle fibres and showed evidence of intercalated discs.⁷⁶ Such satellite cells may withstand a short-term ischaemic episode⁷⁷ and could provide a valuable myocardial repair strategy. Foetal myocardial tissue has also been successfully transplanted into myocardial scar tissue with graft survival from 7 to 24 days after infarction: a duration of viability that may support delivery of a range of therapeutic proteins.⁷⁸ Implantation of neonatal skeletal myocytes into scar tissue resulted in the establishment of new muscle tissue that was capable of contracting when electrically stimulated.⁷⁹ At day 1, grafted cells were proliferating and did not express MHC and by 2 weeks the grafts began expressing β -MHC, a characteristic feature of slow skeletal myocytes. Co-expression of embryonic, fast and β -MHC continued over 3 months, consistent with conversion of the grafts to slow twitch fibres that could prove to be suited to a cardiac work load.

MOLECULAR CARDIOMYOPLASTY

Dynamic cardiomyoplasty is a therapeutic procedure that involves wrapping a synchronously paced skeletal muscle, usually the latissimus dorsi, around the heart.^{80,81} A potentially analogous gene therapy approach to cardiomyoplasty would be to use genetic manipulation to regenerate cardiac myocytes after myocardial infarction. On overexpression, the myogenic determination factor MyoD^{82,83} is capable of inducing skeletal muscle differentiation of a number of cell types, including fibroblasts. A number of recent studies, including our own, have addressed the possibility that forced expression of MyoD could be used for converting non-myocytes within infarcted or scarred myocardial tissue with a positive therapeutic outcome.^{82,84,85} We have demonstrated that overexpression of MyoD through retroviral delivery into primary neonatal cardiac fibroblasts in culture will result in the formation of elongated multinucleated myotubes. Upon delivery of the retrovirus by injection into infarcted dog myocardium, we identified rare clusters of cells that stained positive for skeletal muscle-specific skeletal fast MHC.⁸⁴

In healing rat hearts injured 1 week previously, it was found that cardiac granulation (wound repair) tissue could be successfully

infected with a MyoD-encoding Ad. Infected cardiac granulation tissue expressed MyoD mRNA and there was evidence of structures suggesting multinucleated myotubes.⁸⁵

SUMMARY/CONCLUDING REMARKS

There have been major advances in myocardial gene transfer in recent years, with successful demonstrations of high transfection efficiencies, high-level transgene expression and regulation of foreign gene expression in a manner that would be applicable to gene therapy for the diseased myocardium. The principal methods for foreign gene delivery to the myocardium have involved direct introduction of transgenes into cardiac myocytes and grafting of cardiac myocytes or myoblasts that have been transfected *ex vivo* with a foreign gene. Both direct gene transfer and cell grafting have limitations. Initial studies on direct gene transfer by plasmid or viral vectors resulted in poor transfection efficiencies and a lack of sustained transgene expression in cardiac myocytes. While direct plasmid gene transfer is inefficient, delivery of Ad-based vectors has resulted in inflammatory responses that elicit a shut-off of foreign gene expression. New modified Ad and AAV have been particularly effective for achieving high-level foreign gene expression in cardiac myocytes in the absence of a significant inflammatory reaction. Cellular transplantation may hold promise for repair of compromised myocardial tissue, but its application to gene therapy approaches must await the demonstration of a functional improvement in the myocardium following cell grafting.

A critical requirement for an effective gene therapy vector is the ability to regulate levels of gene expression temporally and spatially in a manner that is appropriate to the disease state. While this could be achieved through hormone or drug-responsive promoters, this would require additional monitoring and exogenous administration of an additional drug that would result in clinical procedures that do not significantly differ from those of conventional medicine. Our recent demonstration of hypoxia-responsive transgene expression in a model of myocardial ischaemia with reperfusion is the first demonstration of regulation of a transgene in response to an endogenous disease phenotype that is fundamental for the development of future successful gene therapy vectors.

As many of the initial concerns and limitations are becoming eliminated, it is clear that foreign genes can be delivered to the myocardium with high efficiency and that appropriate vectors are now available to ensure adequate gene transfer in the absence of significant inflammatory responses. The ability to regulate transgene expression appropriately for a particular disease phenotype will now permit development of new effective viral vectors for clinical applications directed at therapeutically relevant targets.

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EXHIBIT D



THE ANNALS OF THORACIC SURGERY



In vivo gene gun-mediated transduction into rat heart with Epstein-Barr virus-based episomal vectors

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In Vivo Gene Gun–Mediated Transduction Into Rat Heart With Epstein-Barr Virus-Based Episomal Vectors

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Background. Gene guns have been used to transfer genes into various organs, but there has been no report of successful gene gun–mediated gene transfer into the heart. In this study, we assessed the possibility of gene therapy using a gene gun and an episomal plasmid vector.

Methods. Gene transfer was performed using two sizes of gold particles and two plasmids (an episomal vector and a conventional plasmid vector). From the first to eighth week after the bombardment, rats were sacrificed. The excised hearts were subjected to X-gal staining and histologic examination. To ensure that plasmid was not distributed to organs other than the heart, the presence of

the β -gal sequence was examined by polymerase chain reaction analyses.

Results. Gene expression persisted for 6 weeks. The episomal vector apparently contributed to long-lasting expression. Infiltration of monocytes or leukocytes was very faint. The β -gal DNA was detected in bombarded hearts but not other organs.

Conclusions. Gene gun–mediated transfer of the episomal vector into beating heart may provide a simple, efficient, and useful strategy for gene therapy.

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In vivo gene transfer into the beating heart is an attractive strategy for gene therapy for cardiovascular diseases. Many techniques for gene transfer have been reported, including direct injection of naked plasmid DNA and infusion of various viral or nonviral vectors. In this study, we tested the possibility of direct in vivo transfer of plasmid DNA into heart by a gene gun.

In a gene gun system, micron-sized gold particles are coated with plasmid DNA and then accelerated at high velocity toward target cells or tissues. Cells penetrated by these particles have a high likelihood of being transfected by the DNA thus introduced. Gold and tungsten particles are commonly used as the carrier of plasmid DNA. Because of the high specific gravity and small diameter, these particles easily penetrate into cells. Also, they are not cytotoxic. The gene gun was first devised to transfect plant cells, the walls of which act as a physical barrier to conventional transfection techniques [1]. More recently, it was demonstrated that gene transfer into various mammalian tissues could also be successfully achieved by the gene gun. These tissues include liver, skin, skeletal muscle [2], and pancreas [3]. To our knowledge, however, heart has not yet been targeted.

We employed an Epstein-Barr virus (EBV)-based epi-

somal vector to obtain long-lasting transgene expression in vivo. The EBV-based episomal vector is a plasmid vector carrying oriP and the EBV nuclear antigen 1 (EBNA1) gene from EBV. The EBNA1 gene facilitates the maintenance of the episomes through binding to oriP. After being transfected into human cells, the plasmid persists extrachromosomally at low copy numbers owing to replication and nuclear retention of plasmid DNA [4].

We show here that gene gun–mediated transfer of the EBV-based episomal vector into rat heart results in long-lasting expression of a marker gene.

Material and Methods

Animals and Surgery

Male Wistar rats were used for this study. All rats were between 10 and 12 weeks old. They received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (National Institutes of Health publication 86 to 23, revised 1985). After being anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection), rats were intubated and ventilated with room air. An anterolateral thoracotomy was performed at the location of most pronounced cardiac pulsation and beating hearts were exposed. The pericardium was stripped, and the right chest and abdomen were pressed to push the heart out of the thoracic cavity. After bombardment in

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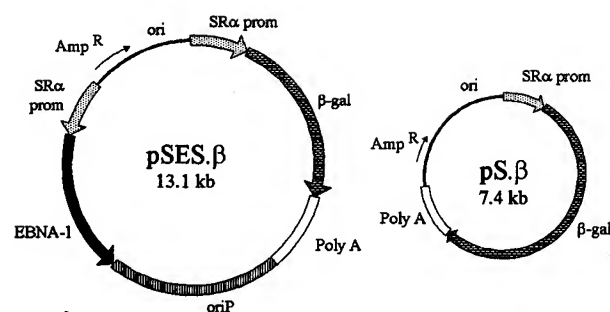


Fig 1. Plasmids used in this study. Maps of pSES.β (left) and pS.β (right) are shown. (Prom = promoter, polyA = SV40 polyA additional signal.)

the anterior wall of left ventricular near the apex, a chest tube was placed to drain air and fluids, and the wound was closed in two layers.

Plasmids

The plasmid vectors, pSES.β and pS.β, were previously described [5]. Briefly, pSES.β (Fig 1, left) is composed of the *Escherichia coli* β-gal gene located between SRα promoter and the SV40 polyA additional signal, EBV oriP, EBV EBNA1 gene under control of the SRα promoter, the ampicillin resistance gene, and the replication origin for *E. coli*. The other plasmid, pS.β (Fig 1, right), was constructed from pSES.β by deleting EBNA1 and oriP.

Bombardment

We used a gene delivery system (model TF-1) designed by Nihon Medical & Chemical Instruments Co, Ltd, (Osaka, Japan). With this device, high-pressure helium provides the motive force for gold particles. The size of gold particles influences the transduction efficiency as well as the degree of tissue damage. Although the larger particles reach the deeper layer, cell damage could be more serious. Therefore, two sizes of gold particles were examined (1.0 Au and 1.5 Au: 1.0 μm and 1.5 μm in diameter, respectively). Supercoiled plasmids were precipitated gold particles at a ratio of 20 μg DNA:5 mg gold particles. The DNA-coated particles were suspended in 500 μL ethanol, and 20 μL of the suspension was distributed on a 25-mm titanium plate (0.5-mm thickness). Each rat received two consecutive bombardments in the anterior wall of left ventricular near the apex. The size of bombarded area was about 0.5 to 1.0 cm². The helium pressure was set at 1.05 MPa, and the distance between the surface of the heart and the titanium plate was 2 cm. As a control group, 10 rats (1.0 Au, n = 5; 1.5 Au, n = 5) were bombarded with uncoated gold particles.

Histologic Analysis

For histologic examination, animals were sacrificed 1, 2, 3, 4, 6, or 8 weeks after bombardment. An 18-gauge catheter was inserted into the abdominal aorta and a median sternotomy was performed. After the pulmonary artery and inferior vena cava were cut, the heart was perfused with 50 mL iced PBS followed by 20 mL 4%

paraformaldehyde (PFA) in a retrograde manner using this catheter. The hearts were excised, sliced (500 μm thick) by a vibratome slicer (DOSAKA, Kyoto, Japan), and refixed in 4% PFA for 1 hour. After washing three times in PBS at room temperature, all sections were incubated overnight at 37°C in X-gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactosidase [X-gal], 3 mmol/L K₄[Fe(CN)₆], 3 mmol/L K₃[Fe(CN)₆], 1 mmol/L MgCl₂, and 0.1% Triton X-100 in PBS). After being washed in PBS, the samples were refixed overnight with 4% PFA at 4°C, dehydrated in ethanol, and embedded in paraffin. We judged whether the bombarded portion of the heart stained blue by microscopic observations.

Polymerase Chain Reaction

For polymerase chain reaction (PCR) testing, DNA was isolated from the lung, brain, liver, spleen, kidney and heart of animals 7 days after bombardment. Synthetic oligodeoxynucleotide primers were prepared corresponding to the β-gal gene sequence (sense primer: 5'-GCC GAC CGC ACG CCG CAT CCA GC-3', anti-sense primer: 5'-CGC CGC GCC ACT GGT GTG GGC C-3') [6]. The reaction mixture consisted of 1 mg DNA, 1.5 mmol/L MgCl₂, 2 mmol/L dNTP, 0.5 μmol/L of each oligonucleotide primer, and 2.5 U of Taq DNA polymerase (Amersham Pharmacia Biotech Ltd, Uppsala, Sweden). As positive controls, 5 ng pS.β and pSES.β were also tested. The reaction was performed by Gene Amp 2400 (Perkin Elmer, Norwalk, CT). The amplification profile consisted of 25 cycles of denaturing at 98°C for 15 seconds, annealing at 65°C for 2 seconds, and extension at 74°C for 30 seconds. PCR products were analyzed by polyacrylamide gel electrophoresis.

Results

Bombardment Did Not Affect Rat Survival

We used 133 rats in this study. All rats survived surgery and bombardment procedures, with the exception of 3 rats in early experiments that died of respiratory failure within a few hours after surgery. The remaining rats were maintained on a normal diet until subsequent steps in experiments.

Prolonged Expression of β-Gal in Heart Transduced With EBV-Plasmid by Gene Gun

Two sizes of gold particles and two plasmids (pSES.β and pS.β) were employed (Fig 1). One hundred and twenty rats were divided into four groups, with each group of rats receiving a different combination of plasmid/gold particle (pS.β/1.5 Au, n = 30; pSES.β/1.5 Au, n = 30; pS.β/1.0 Au, n = 30; and pSES.β/1.0 Au, n = 30). Five rats of each group were sacrificed 1, 2, 3, 4, 6, and 8 weeks after bombardment, and their hearts were subjected to X-gal staining and histologic examination.

Seven days after the bombardment, cardiomyocytes in the bombarded portion in all hearts stained blue (Fig 2). The staining layer in bombarded heart was within 1 mm

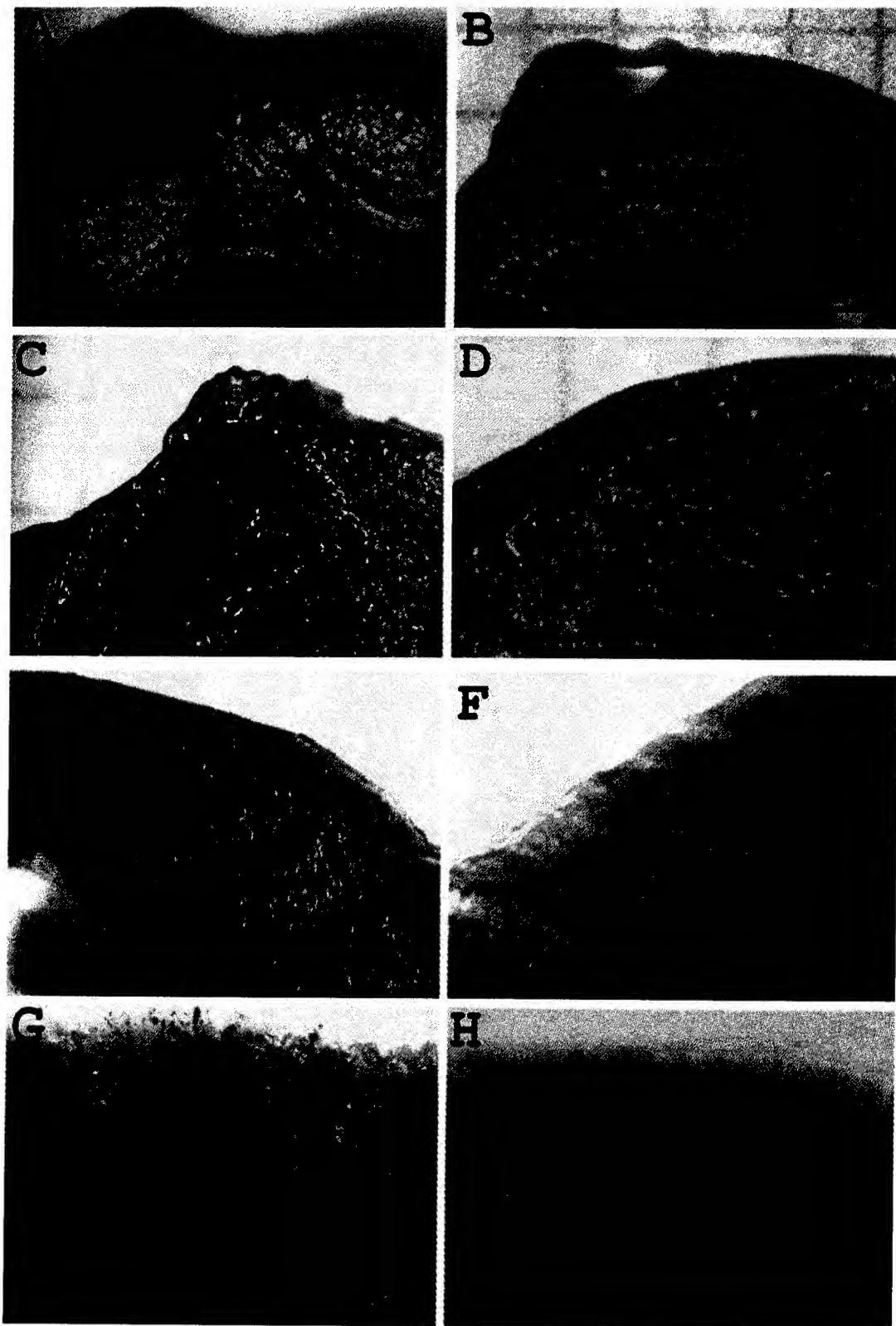


Fig 2. X-gal staining of the bombarded heart. Rat hearts were bombarded with 1.0 Au (A, B, E, and F) or 1.5 Au (C, D, G, and H) gold particles coated with pSES.β (A, C, E, and G) or pS.β (B, D, F, and H). Seven days (A–D) or 4 weeks (E–H) later, rats were sacrificed and the hearts were excised. The hearts were cut into pieces and subjected to X-gal staining as described in Material and Methods. The bar indicates 500 μ m.

Table 1. Period of Gene Expression

Plasmid	Gold Particle	1 Week	2 Weeks	3 Weeks	4 Weeks	6 Weeks	8 Weeks
pSES.β	1.5 Au	5/5 ^a	5/5	5/5	5/5	0/5	0/5
pS.β	1.5 Au	5/5	5/5	5/5	1/5	0/5	0/5
pSES.β	1.0 Au	5/5	5/5	5/5	5/5	5/5	1/5
pS.β	1.0 Au	5/5	5/5	5/5	5/5	1/5	0/5

^a Number of X-gal positive hearts/number of tested hearts.

depth from the surface. Gene expression persisted for 3 weeks (pS.β/1.5 Au), 4 weeks (pSES.β/1.5 Au and pS.β/1.0 Au), or 6 weeks (pSES.β/1.0 Au; Table 1). The hearts of control rats (1.5 Au, n = 5; and 1.0 Au, n = 5) did not stain at all (data not shown). Transfection with an EBV-based episomal vector resulted in more sustained expression of the marker gene than that with a conventional, non-EBV plasmid vector, provided that gold particles of the same size were employed. On the other hand, when the same plasmid was transfected, 1.0 Au was more effective than 1.5 Au, in terms of longevity of gene expression. The combination of 1.0 Au and pSES.β yielded the most prolonged expression in this study. EBV episomal vector apparently contributed to long-lasting expression of β-gal expression.

On histologic examination, many cardiomyocytes in the surface layer stained blue, while few cells stained in deep layers (Fig 2, Fig 3). Probably, DNA-coated gold particles could not reach the deep layers. Some cardiomyocytes carrying DNA-coated gold particles were observed (Fig 3C and D).

Vector DNA Detected in Bombarded Heart but Not in Other Organs

To ensure that plasmid was not distributed to organs other than the heart under the experimental protocol, we

performed PCR and examined the presence of the β-gal sequence. DNA was prepared from the lung, brain, liver, spleen, kidney, and heart of rats in each group sacrificed 7 days after bombardment. The β-gal sequence was detected in bombarded heart but not in other organs or control heart (Fig 4). These findings indicated that the plasmid DNA was present exclusively in the heart.

Comment

In the present study, we showed that plasmid DNA can be introduced into cardiomyocytes using a gene gun and that transfer of the EBV-based episomal vector results in long-lasting gene expression in vivo. To our knowledge, this is the first study showing that in vivo gene transfer into heart can be achieved by a gene gun.

Various gene transfer vectors and delivery methods have been devised to transfer genes into the heart, including direct injection of naked DNA into heart muscle [7], infusion of HVJ-liposomes [8] or cationic liposomes [9] into coronary artery, and injection of HVJ-liposomes into pericardium [10]. Particle-mediated gene transfer has several advantages over other methods. First, the gene gun is a nonviral vector. Side effects associated with viral vectors (eg, generation of aberrantly recombinant, replication-competent viruses) can be avoided. Second, this technique may allow expression of foreign genes in a wide variety of cell types, including terminally differentiated nondividing cells. Third, it can transfer as large a quantity of DNA as can be carried by

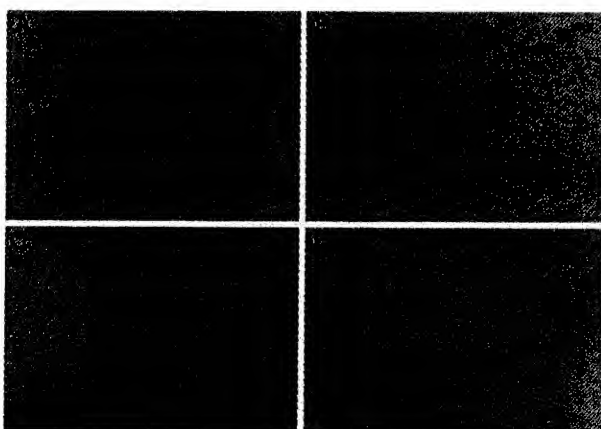


Fig 3. Microscopic observation of bombarded heart stained with X-gal. Rats hearts were bombarded with 1.0 Au (A and B) or 1.5 Au (C and D) gold particles coated with pSES.β (A and C) or pS.β (B and D), and 3 weeks later, rats were sacrificed and the hearts were excised. The heart sections were subjected to X-gal staining and Kernechtrot staining so that nucleus stained pink. The arrow indicates gold particle. Magnification $\times 400$, A–C; $\times 200$, D.



Fig 4. Polymerase chain reaction (PCR) analyses of DNA from various organs. DNA was prepared from the lung (lane 1), brain (lane 2), liver (lane 3), spleen (lane 4), or kidney (lane 5) of a rat whose heart was bombarded with pSES.β/1.0 Au. DNA was also prepared from hearts bombarded with pS.β/1.5 Au (lane 6), pSES.β/1.5 Au (lane 7), pS.β/1.0 Au (lane 8), pSES.β/1.0 Au (lane 9), or 1.0 Au alone (lane 10). As positive controls, 5 ng pS.β (lane 11) and pSES.β (lane 12) were also tested. PCR was performed using β-gal specific primers as described in Material and Methods. (M = molecular weight marker.)

gold particles. Using submicrogram quantities of DNA per bombardment, 1,000 to 10,000 copies of DNA can be delivered into each target cell [11]. Fourth, genes can be introduced exclusively into the bombarded region without being redistributed to other organs.

In particle-mediated transfer, micron-sized gold particles are coated with DNA and accelerated at high velocity toward target cells or tissues. The process may not depend on particular biochemical structure or physical feature of the target cell membrane like liposome/DNA complex-mediated gene transfer [11]. It may be possible to employ a gene gun to transfect cells that are relatively resistant to other delivery systems. For example, it is difficult to transfer genes into infarct areas by coronary infusion, whereas our delivery method may permit perioperative gene transfer into such areas.

In a previous study, we transfected rat cardiac graft *ex vivo* with replication-incompetent adenovirus vector by coronary infusion [12]. After transplantation, massive infiltration of leukocytes was observed close to the transgene-positive cells. It was difficult to avoid the immune responses against the viral vector. In the present study, however, infiltration of monocytes or leukocytes was very faint, if any (Fig 3). No cell damage or inflammatory response was demonstrated by a histologic survey. This is another advantage of gene gun-mediated gene transfer.

We consider that the present method may be useful in treating severe ischemic heart disease by transferring genes to promote angiogenesis, such as basic fibroblast growth factor (bFGF) [13], vascular endothelial growth factor (VEGF) [14], and hepatocyte growth factor (HGF) [15] genes. For patients suffering from severe ischemic heart disease resistant to conventional therapy, transmyocardial laser revascularization (TMLR) is useful. TMLR not only increases the supply of oxygenated blood to the myocardium via left ventricular transmural channels but also induces angiogenesis. If gene gun-mediated transfection of angiogenesis factor genes can be combined with TMLR, angiogenesis may be more efficiently promoted by synergistic action of two systems. Moreover, TMLR may enable gene gun to transfect cells in deeper layers. The combination may greatly contribute to the treatment of patients with various heart diseases, especially severe ischemic heart disease. On the other hand, a gene gun may be equipped on a tip of catheter. Such a device may be feasible for the gene therapy against the coronary stenosis, as well as for endocardial delivery.

We previously demonstrated high transfection efficiency with EBV-based episomal vectors into various human lymphoma cell lines [16-18], hepatocellular carcinoma cell lines [19], primary fibroblasts from skin, bone marrow cells [5], and peripheral blood CD34⁺ cells [20]. Recently, we have also reported that high transient expression was observed in rat heart injected with naked EBV-based plasmid DNA [21]. In the present study, we found that transfection with the EBV-based episomal vector results in more prolonged gene expression in rodent cells than that with conventional plasmid vector.

This is compatible with earlier finding by Saeki and coworkers [22] who reported sustained expression of a marker gene in rat liver injected with EBV-based episomal vector by means of the hemagglutinating virus of Japan (HVJ)-liposome [22]. By our hands, the gene gun was more efficacious than naked plasmid injection. We have two reasons for this high efficiency. First, we employed an episomal plasmid. Second, the gene gun-mediated delivery system enables transfer of the plasmid-coated particles into the nucleus, whereas direct injection of plasmid allows penetration of DNA into cytoplasm but not nucleus.

Direct *in vivo* gene transfer into heart is an attractive strategy for gene therapy. Particle-mediated gene transfer technology provides a physical means of DNA delivery, and the EBV-based episomal vector contributes to stronger and more long-lasting expression. Our findings suggest that the combination of the gene gun and EBV-based vector may be useful for gene therapy of cardiovascular diseases.

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New Requirements for Recertification/Maintenance of Certification in 2001

Diplomates of the American Board of Thoracic Surgery who plan to participate in the recertification/maintenance of certification process in 2001 should pay particular attention to this notice because the requirements have changed.

In addition to an active medical license and institutional clinical privileges in thoracic surgery, effective in 2001, a valid certificate is an absolute requirement for entrance into the recertification/maintenance of certification process. If your certificate expired, the only pathway for renewal of a certificate will be to take and pass the Part I (written) and the Part II (oral) certifying examinations.

In 2001, the American Board of Thoracic Surgery will no longer publish the names of individuals who have not recertified. In the past, a designation of "NR" (not recertified) was used in the American Board of Medical Specialties directories if a Diplomate had not recertified. The Diplomate's name will be published upon successful completion of the recertification/maintenance of certification process.

The CME requirements have also changed. The new CME requirements are 70 Category I credits in either cardiothoracic surgery or general surgery earned during the 2 years prior to application. SESATS and SESAPS are the only self-instructional materials allowed for credit. Category II credits are not allowed. The Physicians Recognition Award for recertifying in general surgery is not allowed in fulfillment of the CME requirements. Interested individuals should refer to the 2001 *Booklet of Information* for a complete description of acceptable CME credits.

Diplomates should maintain a documented list of their major cases performed during the year prior to application for recertification. This practice review should consist of 1 year's consecutive major operative experiences. If more than 100 cases occur in 1 year, only 100 should be listed.

Candidates for recertification/maintenance of certification will be required to complete both the general thoracic and the cardiac portions of the SESATS self-assessment examination. It is not necessary for candidates to purchase SESATS individually because it will be sent to candidates after their application has been approved.

Diplomates may recertify the year their certificate expires, or if they wish to do so, they may recertify up to two years before it expires. However, the new certificate will be dated 10 years from the date of expiration of their original certificate or most recent recertification certificate. In other words, recertifying early does not alter the 10-year validation.

Recertification/maintenance of certification is also open to Diplomates with an unlimited certificate and will in no way affect the validity of their original certificate.

The deadline for submission of applications for the recertification/maintenance of certification process is May 1 each year. A brochure outlining the rules and requirements for recertification/maintenance of certification in thoracic surgery is available upon request from the American Board of Thoracic Surgery, One Rotary Center, Suite 803, Evanston, IL 60201; telephone number: (847) 475-1520; fax: (847) 475-6240; e-mail: abts@evanston@msn.com.

In vivo gene gun-mediated transduction into rat heart with Epstein-Barr virus-based episomal vectors

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Ann Thorac Surg 2000;70:1332-1337

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ACQUIRED DISEASES

RESEARCH ARTICLE

Direct intra-cardiomuscular transfer of β_2 -adrenergic receptor gene augments cardiac output in cardiomyopathic hamsters

K Tomiyasu¹, Y Oda¹, M Nomura², E Satoh², S Fushiki³, J Imanishi², M Kondo¹ and O Mazda²

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In chronic heart failure, down-regulation of β -adrenergic receptor (β -AR) occurs in cardiomyocytes, resulting in low catecholamine response and impaired cardiac function. To correct the irregularity in the β -AR system, β -AR gene was transduced *in vivo* into failing cardiomyocytes. The Epstein–Barr virus (EBV)-based plasmid vector carrying human β_2 -AR gene was injected into the left ventricular muscle of Bio14.6 cardiomyopathic hamsters whose β -AR is down-regulated in the cardiomyocytes. The echocardiographic examinations revealed that stroke volume (SV) and cardiac output (CO) were significantly elevated at 2 to 4 days after

the β_2 -AR gene transfer. Systemic loading of isoproterenol increased the cardiac parameters more significantly on day 2 to day 7, indicating that the adrenergic response was augmented by the genetic transduction. The same procedure did not affect the cardiac function of normal hamsters. Immunohistochemical examinations demonstrated human β_2 -AR expression in failing cardiomyocytes transduced with the gene. RT-PCR analysis detected mRNA for the transgene in the heart but not in the liver, spleen, or kidney. The procedures may provide a feasible strategy for gene therapy of severe heart failure. Gene Therapy (2000) 7, 2087–2093.

Keywords: heart failure; β -adrenergic receptor; Epstein–Barr virus-based-plasmid vector; cardiomyopathic hamster; gene therapy

Introduction

In chronic cardiac failure, persistent excitation of the sympathetic nervous system ultimately leads to down-regulation of the β -AR in cardiac cells, contributing to the decrease in cardiac function. If a large number of β -AR can be expressed in cardiomyocytes through genetic transduction technique, reactivity to catecholamines may be improved, enabling the possible recovery of cardiac contractility.^{1–3} Milano *et al*⁴ established a transgenic mouse constitutively expressing β_2 -AR in the heart. They found a significant increase in the heart rate (HR), as well as improvement in the dp/dt max, an index of isochronic contractility. More recently, Kawahira *et al*⁵ and Kypson *et al*⁶ transduced the β_2 -AR gene *ex vivo* into donor hearts isolated from pressure-overloaded and normal rats, respectively. They found elevated LV dp/dt max in the heart graft heterotopically transplanted into recipient animals. Maurice *et al*⁷ transduced a normal rabbit heart *in vivo* with the β_2 -AR gene resulting in the elevation of LV dp/dt max. Although these earlier reports strongly suggest the feasibility of the β_2 -AR gene transfer in terms of gene therapy for heart failure, there have been no reports showing *in vivo* transduction of the β_2 -AR gene into failing hearts, with attendant cardiac functional restoration.

To express a large number of β_2 -AR in failing cardiac muscle cells, it is necessary to find ways to conduct genetic transduction as efficiently as possible. Previously, we reported that a high rate of gene transduction could be achieved in cardiomyocytes *in vivo* through injection of the Epstein–Barr virus (EBV)-based plasmid.⁸ In the present study, we used this method to transduce the β_2 -AR gene into the hearts of cardiomyopathic hamsters. Because direct injection of naked plasmid DNA allows transgene expression specifically in cardiac muscle, and employment of the EBV-based plasmids enables highly efficient gene transduction, our system is considered to be highly appropriate for gene therapy of heart failure.

Results

We first estimated the transduction efficiency *in vivo* into cardiac muscle using our system. pSES. β (Figure 1) was inoculated into the hearts of the F1b hamsters, and X-gal staining was performed. The β -gal expression was demonstrated in cardiac muscle within approximately 0.1 cm from each injection site (Figure 2a). This is compatible with our previous finding using normal rat heart.⁸

Next, the Bio14.6 hamsters were injected with pSES. β_2 -AR or pSES. β into the left ventricles, and their cardiac function was evaluated before and 2 and 4 days after the transduction. Figure 3 and Table 1 summarize the cardiac function parameters obtained by the echocardiographic analyses. The hamsters transduced with pSES. β_2 -AR gene showed 1.6-fold higher cardiac output (CO) than the

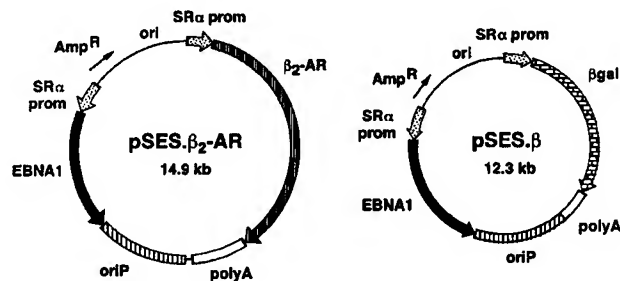


Figure 1 Plasmids used in this study. Maps of pSES.β₂-AR (left) and pSES.β (right)⁶⁷ are shown. Prom, promoter; polyA, SV40 polyA additional signal.

pSES.β-transduced animals on day 4 after the gene transfer (45.6 ± 21.0 ml *versus* 70.8 ± 21.3 ml, $P < 0.05$) (Figure 3, left lower panel). Cardiac response to isoproterenol, a β-AR agonist, was also studied by systemic loading of the drug. On day 4, pSES.β₂-AR-transduced group showed 1.5-, 1.3- and 1.5-fold higher value for fractional shortening (FS), stroke volume (SV) and CO, respectively, in comparison with the pSES.β-transduced group (FS: 22.7 ± 1.5 ml *versus* 14.9 ± 5.7 ml, $P < 0.05$; SV: 0.256 ± 0.048 ml *versus* 0.204 ± 0.081 ml, $P < 0.05$; CO: 95.8 ± 20.7 ml *versus* 63.5 ± 31.8 ml, $P < 0.05$) (Figure 3, right panels). In the group given pSES.β₂-AR, the FS was 1.3-fold higher than that obtained before transduction. (FS: 22.7 ± 1.5 ml *versus* 17.0 ± 2.9 ml, $P < 0.05$) (Figure 3, right upper panel). The SV and CO of the gene-transduced animals were elevated 1.7- and 1.8-fold on day 2, respectively, in comparison with the levels before the genetic transduction (SV: 0.296 ± 0.023 ml *versus* 0.170 ± 0.037 ml, $P < 0.005$; CO: 112.3 ± 16.7 ml *versus* 62.9 ± 13.3 ml, $P < 0.005$) (Figure 3, right middle and lower panels). The results indicate that intramyocardial injection of pSES.β₂-AR elicits improvement in cardiac function, as well as augmentation of adrenergic response in the cardiomyopathic hamsters.

To examine the influence of gene transduction on cardiac function of normal animals, the F1b hamsters were also tested. Compared with Bio14.6, the F1b hamsters show smaller SV and CO, due to smaller left ventricular internal dimension at end-diastole (Dd) and left ventricular internal dimension at end-systole (Ds) (Tables 1 and 2). When the F1b were transduced with pSES.β₂-AR or pSES.β, no significant change was observed in the parameters before and subsequent to genetic transduction, regardless of the plasmid injected (Table 2). Systemic administration with isoproterenol showed that the adrenergic response in pSES.β₂-AR-transduced F1b was comparable to that in F1b given transduction with control plasmid.

Next, the parameters for cardiac function were computed for the Bio14.6 animals that received pSES.β₂-AR or pSES.β transduction, and kinetic changes were monitored up to the 9th day following transduction. In the group transduced with pSES.β₂-AR, significant elevation in the SV and CO was seen on day 2 to 7, compared with the levels before genetic transduction (Figure 4).

The human β₂-AR was demonstrated in cardiomyocytes from the pSES.β₂-AR-transduced Bio14.6 by immunohistochemical staining (Figure 2b). In contrast, the hearts of pSES.β-transduced Bio14.6 did not stain

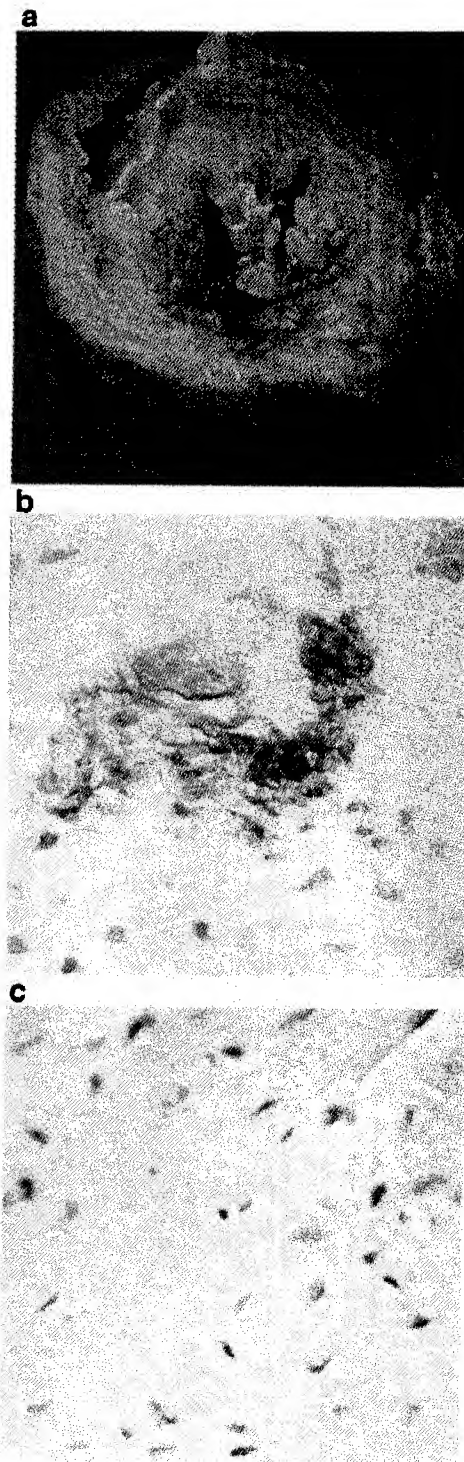


Figure 2 Gene transfer *in vivo* by intra-myocardial injection of plasmid DNA. (a) Three hundred μg of pSES.β was injected into the left ventricle of F1b, and 5 days later, the animal was killed. The heart was fixed and stained with X-gal. (b and c) Three hundred μg of pSES.β₂-AR (b) or pSES.β (c) was injected into the left ventricle of the Bio14.6, and 5 days later, the animal was killed. A frozen section of the heart was immunostained with anti-human β₂-AR antibody.

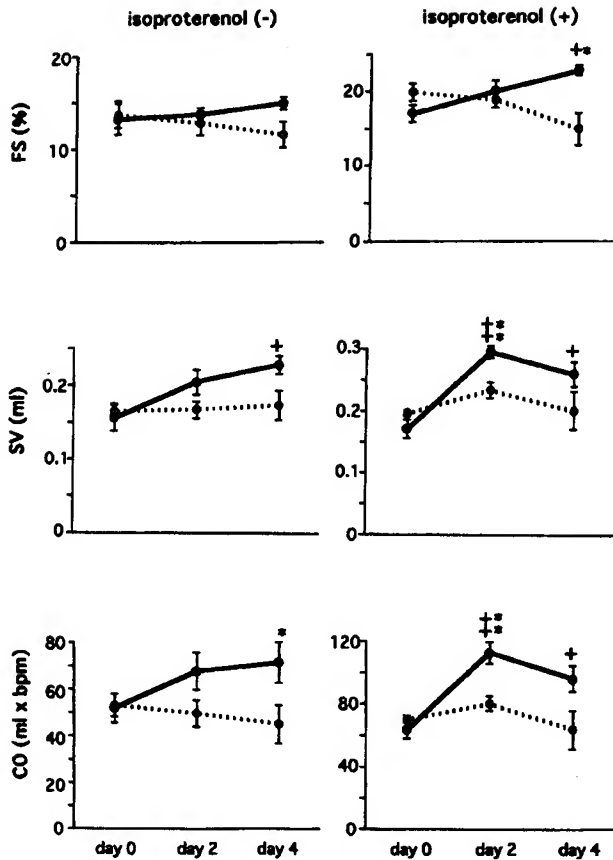


Figure 3 Improvement of cardiac function of gene-transduced Bio14.6 cardiomyopathic hamsters. The Bio14.6 were transduced with pSES.β₂-AR (solid lines, n = 7) or pSES.β (dotted lines, n = 6). On the indicated days after the transduction, echocardiographic examination was performed before (left panels) or 5 min after (right panels) percutaneous administration of 0.1 mg/kg of isoproterenol. Results are expressed as mean ± s.d. (+: P < 0.05, ++: P < 0.005 in comparison with the parameters for pre-transduction; *: P < 0.05, **: P < 0.005 in comparison between pSES.β₂-AR-transduced and pSES.β-transduced groups).

with the anti-human β₂-AR antibody that does not recognize hamster β₂-AR (Figure 2c). RT-PCR experiments demonstrated the transgene-specific mRNA in pSES.β₂-AR-injected heart, but not in liver, kidney or spleen of the same animal (Figure 5). The sense and antisense PCR primers were set corresponding to SRα and β₂-AR sequences, respectively, so that endogenous β₂-AR mRNA is not amplified. The results indicate that the transgene expression was achieved exclusively in the heart by intra-myocardial injection of the naked EBV-based plasmid vector.

Discussion

In the present study, we succeeded in augmenting cardiac function in cardiomyopathic hamsters by transfecting β₂-AR gene into the cardiac muscle, in an effort to correct irregularities in the β-AR system of failing cardiomyocytes. Administration with isoproterenol resulted in an even more significant improvement in cardiac function. The increase in CO may be ascribed to an increase in the SV rather than that in the HR. As far as we know,

there have been no reports showing *in vivo* genetic transduction into failing cardiomyocytes.

Unlike the β₂-AR transgenic mouse, the β₁-AR transgenic mouse did not show elevated cardiac contractility.^{9,10} Therefore, the β₂-AR gene may be more suitable for gene therapy of heart failure than the β₁-AR gene.

The *in vivo* genetic transduction into heart tissue has been done using many different methods, including direct intra-myocardial injection of naked plasmid DNA¹¹ and adenoviral vector,¹² and intra-coronary infusion of adenoviral,¹³ HVJ-liposome¹⁴ and AAV¹⁵ vectors. We chose the direct intra-muscular injection method so that the gene expression is limited exclusively to cardiac muscle. Actually, we did not detect any evidence for transgene expression in organs other than the heart by RT-PCR (Figure 5). Another advantage of this method is simplicity and safety. Unlike healthy animals, the physiological data of emaciated animals like cardiomyopathic hamsters fluctuate considerably with even a small degree of invasion, and for this reason, it is necessary to minimize the amount of invasion as much as possible. Provided that direct injection is carried out under echocardiography guidance, *in vivo* naked plasmid injection can be done percutaneously without thoracotomy, so that the degree of invasion with this method is very low. On the other hand, direct injection to cardiac muscle is advantageous over the intra-coronary approach in that the former is also considered appropriate for genetic transduction to cells in an ischemic lesion barely supplied with blood flow. When cardiac incompetence caused by ischemia is considered as a target disease for gene therapy, this point may become important.

An impediment to naked plasmid injection is that the intensity of gene expression is comparatively low. To overcome this problem, we used the EBV-based plasmid vector, which carries oriP and EBNA1 gene from EBV genome. The EBNA1-oriP system facilitates nuclear localization of the plasmid, anchorage of plasmid to nuclear matrix, and transcriptional up-regulation. Previously, we have reported the effectiveness of the vectors in transfecting various cells of human origin both *in vitro* and *in vivo*.^{8,16-20} We also reported that by using the EBV-based plasmid vectors, *in vitro* transient expression over 10 times higher than that by using conventional plasmids was observed, not only in primate cells but in various types of rodent cells as well.⁸ Furthermore, by directly injecting the EBV-plasmid into rat heart, higher rates of genetic transduction and expression were obtained than by injecting conventional plasmids.⁸

With naked plasmid injection, genetic transduction is only possible in a limited portion of the heart. We cautiously injected a small amount of a DNA solution into five locations of the heart, ie the front, rear, and side walls of the left ventricle, the septum, and the apex of the heart, to achieve gene expression over a comparatively wide range of cardiac muscle (Figure 2a). On the other hand, cardiomyocytes are connected by the gap junctions, so that adjacent cells are biochemically aligned and function cooperatively. It has been reported that cAMP, the second messenger mediating adrenergic stimulation, can be transmitted through gap junctions.²¹⁻²³ Taking advantage of this cell-to-cell communication, it may be possible to increase the contractility of cardiac cells which fail to be transduced, but which are connected to neighboring

Table 1 Cardiac function parameters of gene-transduced animals under isoproterenol(-)

Hamster transfection	Bio 14.6					F1b				
	pSES- β (n = 6)					pSES- β 2AR (n = 7)				
Days	0	2	4	0	2	4	0	2	4	0
HR, bpm	317 ± 38	290 ± 56	246 ± 73	329 ± 20	325 ± 39	308 ± 64	370 ± 44	323 ± 43	368 ± 56	407 ± 14
FS, %	13.7 ± 3.8	12.7 ± 3.4	11.4 ± 3.6	13.2 ± 4.1	13.7 ± 1.9	14.8 ± 1.7	45.8 ± 4.7	37.6 ± 6.2	39.0 ± 2.9	34.5 ± 6.4
Dd, mm	7.8 ± 0.4	8.0 ± 0.5	8.2 ± 0.4	7.7 ± 0.7	8.2 ± 0.5	8.2 ± 0.3	4.5 ± 0.2	5.0 ± 0.3	5.2 ± 0.6	4.9 ± 0.5
Ds, mm	6.7 ± 0.6	7.0 ± 0.7	7.2 ± 0.4	6.7 ± 0.7	7.0 ± 0.4	6.9 ± 0.4	2.5 ± 0.3	3.2 ± 0.4	3.2 ± 0.3	3.1 ± 0.4
SV, ml	0.167 ± 0.028	0.171 ± 0.029	0.174 ± 0.053	0.156 ± 0.041	0.203 ± 0.04	0.228 ± 0.029	0.077 ± 0.010	0.095 ± 0.021	0.106 ± 0.036	0.087 ± 0.027
CO, ml × bpm	53.0 ± 12.9	49.1 ± 14.8	45.6 ± 21.0	51.6 ± 15	67.2 ± 19.3	70.8 ± 21.3	28.7 ± 3.2	31.0 ± 8.2	45.5 ± 13.0	35.5 ± 10.5

Values are mean ± s.d.

Table 2 Cardiac function parameters of gene-transduced animals under isoproterenol(+)

Hamster transfection	Bio 14.6					F1b				
	pSES- β (n = 6)					pSES- β 2AR (n = 7)				
Days	0	2	4	0	2	4	0	2	4	0
HR, bpm	358 ± 29	345 ± 47	306 ± 66	370 ± 16	382 ± 45	372 ± 49	420 ± 17	389 ± 20	424 ± 31	448 ± 12
FS, %	19.9 ± 3.2	18.9 ± 2.8	14.9 ± 5.7	17 ± 2.9	20 ± 3.3	22.7 ± 1.5	53.2 ± 3.6	54.8 ± 6.4	55.4 ± 5.3	53.3 ± 4.5
Dd, mm	7.9 ± 0.3	7.9 ± 0.4	8.1 ± 0.5	7.4 ± 0.6	8.3 ± 0.3	7.8 ± 0.5	4.2 ± 0.3	4.3 ± 0.3	4.1 ± 0.5	4.6 ± 0.5
Ds, mm	6.0 ± 0.5	6.4 ± 0.5	6.9 ± 0.7	6.2 ± 0.6	6.5 ± 0.5	6.1 ± 0.4	2.0 ± 0.3	1.9 ± 0.3	1.8 ± 0.3	2.2 ± 0.4
SV, ml	0.197 ± 0.014	0.233 ± 0.033	0.204 ± 0.081	0.17 ± 0.037	0.296 ± 0.023	0.256 ± 0.048	0.067 ± 0.011	0.071 ± 0.014	0.063 ± 0.274	0.087 ± 0.029
CO, ml × bpm	70.5 ± 6.9	79.9 ± 12.9	63.5 ± 31.8	62.9 ± 13.3	112.3 ± 16.7	95.8 ± 20.7	27.9 ± 5.3	28.0 ± 6.1	27.7 ± 11.7	39.2 ± 13.3

Values are mean ± s.d.

cells that are successfully transduced with the β_2 -AR gene.

In our experiments, no significant change was observed in cardiac function of normal hamsters, while Kypson *et al*⁶ and Maurice *et al*⁷ reported significant elevation in LV-dp/dt max in normal animals given adenovirus vector-mediated transduction of β_2 -AR gene. Because our method involved plasmid injection to cardiac muscle, the intensity of β -AR expression may be lower than that in their experiments. Alternatively, the discrepancy may be ascribed to the different parameters measured as an index of cardiac function. We analyzed cardiac function of animals less invasively and more physiologically using echocardiography,²⁴ while both Kypson *et al*,⁶ Maurice *et al*⁷ and Kawahira *et al*⁵ measured LV-dp/dt max by means of cardiac catheterization.

A variety of therapeutic approaches have been devised to augment the β -adrenergic signaling to compensate cardiac function in heart failure (eg administration with dobutamine,²⁵ prenalterol,²⁶ xamoterol,²⁷ and milrinone²⁸). These therapeutic strategies actually improve clinical symptoms during short periods following administration, but fail to significantly prolong the

survival of patients. This may be ascribed to β -AR down-regulation in failing cardiomyocytes. Therefore, β -AR gene transfer may be advantageous over pharmacologic interventions. In contrast, β -AR blockade was revealed to be potentially useful for long-term treatment.^{29,30} The concepts are compatible with the manifestations of the transgenic animals overexpressing the myocardial Gs α , a component of β -AR signaling pathway.³¹ In cases of chronic severe heart failure beyond conventional medical treatment, temporary recovery of cardiac function is mandatory. Enhancing cardiac function by a β -AR increase is potentially the treatment of choice. β -AR gene therapy may be advantageous in terms of improvement of quality of life in acute crisis of chronic heart failure, under enhanced sympathetic activation. Short-term adrenergic stimulation may be effective in weaning from large-dose catecholamine therapy, as well as the introduction of β -AR blockade therapy in severe cases. The therapy may also be applicable to temporal restoration of severe heart failure in patients to be treated by heart transplantation.

In conclusion, improved cardiac function was observed as a result of *in vivo* β_2 -AR genetic transduction in an animal model with failing heart. This suggests new possibilities in molecular treatment of severe cardiac failure in which there are obstacles to the β -AR system, and which are resistant to conventional medical treatment.

Materials and methods

Plasmids

pSES. β_2 -AR and pSES. β are plasmid vectors containing the expression units for the human β_2 -AR cDNA (*EcoRI*-

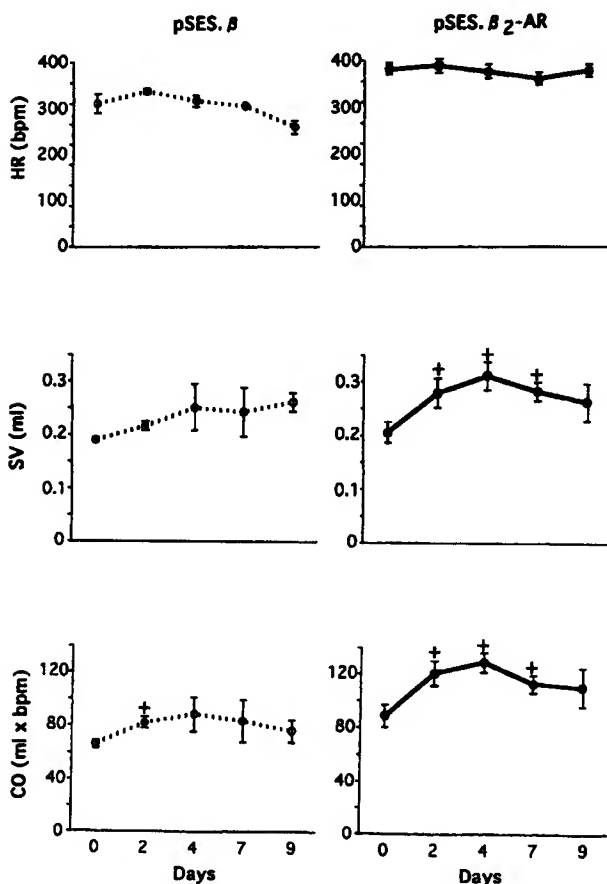


Figure 4 Kinetic change in cardiac function of gene-transduced Bio14.6 cardiomyopathic hamsters. The Bio14.6 were transduced with pSES. β_2 -AR (right panels, $n = 7$) or pSES. β (left panels, $n = 3$). On the indicated days after the transduction, echocardiographic examination was performed 5 min after percutaneous administration of 0.1 mg/kg of isoproterenol. Results are expressed as mean \pm s.d. (*: $P < 0.05$ in comparison with pre-transduction.)

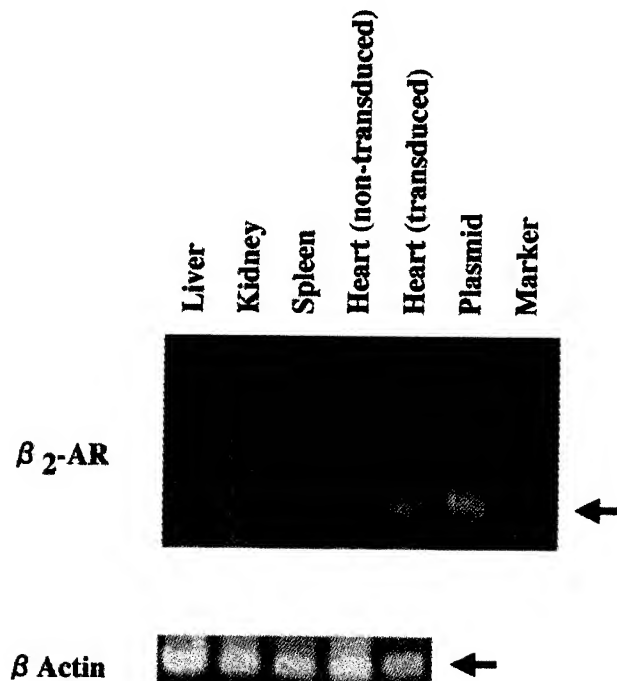


Figure 5 Transgene is exclusively expressed in the heart. The Bio14.6 were transduced with pSES. β_2 -AR, and 10 days later, RNA was extracted from the indicated organs. RT-PCR was performed using the combinations of SR α -specific sense and human β_2 -AR gene-specific antisense primers (upper panel) or β -actin gene-specific sense and antisense primers (lower panel).

Sall 1.8 kb fragment from pTZ β_2 -AR kindly provided by Dr M Bouvier³²⁻³⁴ and *E. coli* β -gal gene, respectively. Both plasmids also possess the EBV oriP element, as well as the EBV nuclear antigen 1 (EBNA1) gene (Figure 1).

Animals

Bio14.6 (cardiomyopathic hamsters) and F1b (normal golden hamsters) were purchased from Charles River Laboratories (Wilmington, MA, USA). As Bio14.6 grow older, they show manifestations of heart failure, β -ARs being down-regulated in the cardiomyocytes.³⁵ The FS of the Bio14.6 were 46.8 ± 4.17 and 13.46 ± 3.78 at 29 and 45 weeks of age, respectively, while that of the 45-week-old F1b was 40.78 ± 7.86 . Therefore, we used 45-week-old Bio14.6 for the experiments.

Gene transfer

The animals were anesthetized by intra-peritoneal injection with sodium thiobarbital (0.1 mg/kg). A DNA/20% sucrose solution (2 μ g/ μ l) containing a small amount of carbon particles as a marker was percutaneously injected into the left ventricular wall, using a microsyringe with a 27G needle, under guidance of the echocardiography (Digital Sonolayer PowerVision SSA-380A equipped with a 7.5-MHz transducer; Toshiba, Tokyo, Japan). Approximately 40 μ l of the solution was injected at each of five locations.

Measurement of cardiac function

The animals were anesthetized by intra-peritoneal injection with sodium thiobarbital (0.1 mg/kg) and analyzed by blinded observers. The HR, FS, Dd and Ds parameters were individually measured by the echocardiographic device before or 5 min after percutaneous administration of 0.1 mg/kg of isoproterenol. The SV and CO were calculated according to following formula:

$$SV = Dd^3 - Ds^3$$

$$CO = SV \times HR$$

Statistical analysis

Differences in cardiac function data were assessed by use of a two way repeated-measures ANOVA. If a significant F ratio was obtained, further analysis was carried out with Fisher's PLSD *post hoc* test. Differences between groups were considered significant at $P < 0.05$.

X-gal staining

The F1b were killed on day 5 following pSES. β_2 transduction. The hearts were excised, cut into slices and fixed with 2% paraformaldehyde for 4 h at 4°C. Following thorough rinsing with PBS, the slices were incubated in an X-gal staining solution (0.05% (v/v) 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) (Nacalai Tesque, Kyoto, Japan), 1 mM MgCl₂, 150 mM NaCl, 3 mM K₄[Fe(CN)₆], 3 mM K₃[Fe(CN)₆], 60 mM Na₂HPO₄, 40 mM NaH₂PO₄ and 0.1% Triton X-100) for 3 h at 37°C.

β_2 -AR immunohistochemistry

The Bio14.6 were killed on day 5 after pSES. β_2 -AR transduction. The hearts were excised, embedded in paraffin, and sliced to a thickness of 5 μ m. The slices were then incubated with an appropriate dilution of anti-human β_2 -AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by rinsing with PBS and further incu-

bation with peroxidase-labeled goat anti-rabbit immunoglobulin (EnVision; Dako, Carpinteria, CA, USA). The β_2 -AR expression was visualized by treatment with DAB solution (Dako).

RT-PCR

The organs were removed from the dead animals and rinsed with PBS. RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol, and RT-PCR was performed as described elsewhere.³⁶ The combinations of SR α -specific sense and β_2 -AR gene-specific antisense primers (5'-CCGCC TGTGGTGCCTCCTGA and 5'-TGCCCATGCCACCA CCCAC, respectively) or β -actin gene-specific sense and antisense primers (5'-GTGCTATCCCTGTACGCCTC and 5'-AGTCCGCCTAGAAGCATTTG, respectively) were used (Figure 1).

Acknowledgements

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EXHIBIT F

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Original Articles: Cardiovascular

Long-Term Gene Transfer in Porcine Myocardium After Coronary Infusion of an Adeno-Associated Virus Vector

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Abstract

Background. Viral vector-mediated gene transfer into the heart represents a potentially powerful tool for studying both cardiac physiology as well as gene therapy of cardiac disease. We report here the use of a defective viral vector, which expresses no viral gene products, for gene transfer into the mammalian heart. Previous studies have used recombinant viral vectors, which retained viral genes and yielded mostly short-term expression, often with significant inflammation.

Methods. An adeno-associated virus vector was used that contains no viral genes and is completely free of contaminating helper viruses. The adeno-associated virus vector was applied to rat hearts by direct intramuscular injection; adeno-associated virus was also infused into pig hearts in vivo via percutaneous intraarterial infusion into the coronary vasculature using routine catheterization techniques.

Results. Gene transfer into rat heart yielded no apparent inflammation, and expression was observed for at least 2 months after injection. Infusion into pig **circumflex** coronary arteries resulted in successful transfer and expression of the reporter gene in cardiac myocytes without apparent toxicity or inflammation; gene expression was observed for at least 6 months after infusion.

Conclusions. We report the use of adeno-associated virus vectors in the cardiovascular system as well as successful myocardial gene transfer after percutaneous coronary artery infusion of viral vectors in a large, clinically relevant mammalian model. These results suggest that safe and stable gene transfer can be achieved in the heart using standard outpatient cardiac catheterization techniques.

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Introduction

Gene therapy has great potential for influencing the natural course of many cardiovascular diseases. One of the most important issues facing practitioners of gene therapy is the choice of vehicle used for gene transfer. Modified viruses have become popular as vectors due to their high efficiency of receptor-mediated cellular uptake and efficient transfer of genetic material into the appropriate subcellular site. Retroviruses, which contain RNA as the genetic material, were historically the first viruses used as gene transfer vectors. Synthesis of a DNA copy of the RNA genome in target cells is necessary before expression of the desired gene. Because most nondividing cells do not permit efficient DNA synthesis, application of these vectors has concentrated on applications for dividing cells such as tumor therapy or modification of dividing cells in tissue culture before transplantation. Viruses with DNA genomes have become widely used for in vivo gene therapy, because the absence of a requirement for DNA synthesis permits gene transfer into nondividing as well as dividing cells with high efficiency.

For cardiovascular gene therapy, efforts have concentrated primarily on the use of adenovirus-based vectors for genetic modification of either liver cells or vascular endothelium [1, 2]. Adenoviral vectors have been used for gene transfer into myocardium of small mammals by direct, intramuscular injection [3, 4] and via infusion of vectors into the coronary arteries of rabbit [5]. Successful gene transfer has also been achieved after direct intramuscular injection of adenoviral vectors into myocardium of large mammals [6, 7]. These studies have found that foreign gene expression was often transient, and a significant cell-mediated immune response was observed [6, 7]. This has led to a search for improved vector systems.

Adeno-associated virus (AAV) vectors represent another approach to cardiovascular gene therapy. Adeno-associated virus is a defective DNA virus, which in nature requires coinfection by adenovirus for efficient replication and spread. Defective viral vectors contain no viral genes but permit packaging of foreign genes into a viral coat. This allows high-efficiency uptake and transfer of foreign genes into target cells while eliminating any immunologic or toxic side effects due to expression of viral genes (Fig 1A). To generate AAV vectors, the foreign gene of interest is flanked by two AAV termini containing only recognition signals for replication and packaging into an AAV coat [8, 9]. All viral genes capable of expressing viral proteins are thereby eliminated from the vector. By contrast, adenoviral vectors are among the class of recombinant vectors in which the gene of interest is inserted in the viral chromosome and one or more genes are removed to prevent viral reproduction. Although pathology due to replication is eliminated using recombinant vectors, the vast majority of viral genes remain (see Fig 1B). As a result, certain viral proteins will be expressed in target cells using current-generation adenoviral vectors [10]. Improved adenoviral vectors have been created in which several potentially toxic genes are eliminated [11]; here we report an adenovirus-based vector that does not contain any viral genes.

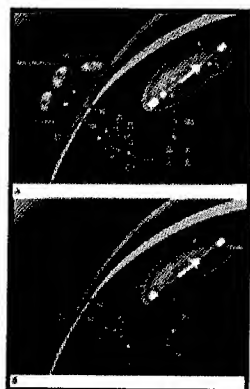


Fig 1. (A) Myocardial gene therapy via adenovirus vectors. The majority of adenovirus vectors contain deletions within one or more essential genes that prevent viral replication and spread, thereby limiting toxicity by this mechanism. Recognition signals for DNA replication and packaging are present (ori, pac); however, numerous genes encoding viral proteins also remain. First, the vector DNA enters the nucleus of the cell (A); messenger RNAs for both the gene of interest (G) as well as certain residual viral genes (V) are synthesized in the nucleus and transported to the cytoplasm (B), which are then translated into proteins (C). The gene of interest may then perform the desired cellular function. As with most cellular proteins, the viral proteins attach to proteins from the major histocompatibility complex (M) for presentation on the cell surface to the immune system (D); cellular proteins are recognized as "self" whereas the viral proteins are recognized as foreign resulting in inflammatory/immune cells recruitment. (B) Myocardial gene therapy via adeno-associated virus vectors. Adeno-associated virus vectors possess only recognition signals for DNA replication and packaging (ori/pac); however, there are no genes remaining that encode viral proteins. Therefore, when the vector DNA enters the cell nucleus (A), only RNA for the gene of interest is synthesized (B), resulting in production of the desired protein in the cytoplasm (C). Because no viral proteins are present for presentation by major histocompatibility complex (M) proteins, the

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target cell is not seen as possessing foreign proteins and no inflammatory/immune cells are recruited.

The AAV vector contains no viral genes and is completely free of contaminating helper viruses. Adeno-associated virus replication and structural proteins are provided by a helper plasmid, which contains the necessary AAV genes but no recognition signals for replication/packaging [9, 12]. This promotes replication and packaging of the vector in tissue culture, but the inability package itself results in elimination of the helper plasmid from the resulting vector stock [9, 12]. Although coinfection by adenovirus is required to permit efficient expression of AAV genes, the contaminating adenovirus in the final product can be completely eliminated, resulting in a pure stock of fully defective AAV vectors [9, 12]. Any basis for toxicity or inflammation in target tissue due to viral gene expression is thereby eliminated with this system. The AAV vector has been used in the hematopoietic system [13] and in the central nervous system [14] to achieve long-term expression of therapeutic genes.

This article describes the use of AAV vectors for gene transfer into mammalian myocardial cells. Adeno-associated virus-mediated gene transfer was achieved in rat myocardial cells in vivo after direct intramuscular injection into the left ventricle. We have also used the AAV vector to achieve successful transfer of a foreign gene into porcine myocardial cells in vivo after infusion into the circumflex coronary artery using selective coronary catheterization. Gene expression within muscle cells of the left ventricle was observed at 3 days, 2 months, and 6 months after vector infusion.

Material and Methods

Plasmids and Viral Vectors

Construction of the plasmid pAAVlac has been described previously [14]. This plasmid contains a transcription unit consisting of the bacterial *lacZ* gene under the control of the human cytomegalovirus immediate-early promoter, as well as a downstream signal for messenger RNA polyadenylation from simian virus 40. The transcription unit is flanked by AAV terminal repeats containing the requisite replication and packaging signals. This plasmid served as the genome for the resulting defective AAV vectors used in the rodent studies. A similar construct was used for the pig studies; however, this vector additionally contained an SV40 splicing signal between the cytomegalovirus promoter and the *lacZ* gene [15]. The method for generation and propagation of packaged AAVlac, adenovirus inactivation, and purification of AAVlac have been described elsewhere [9, 12, 14, 15].

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Direct Injection Into Rat Heart In Vivo

Male Sprague-Dawley rats were used for all studies. Animals were first anesthetized as described previously [16]. A 22-gauge needle on a Hamilton syringe containing 5 μ L of AAV stock (2×10^6 particles/mL) was then introduced by a transthoracic approach into the apex of the left ventricle. When the needle bounced at the approximate heart rate of the subject, the needle was believed to have entered the left ventricular wall. The needle was inserted slightly past this point and the plunger was withdrawn. An absence of blood indicated that the ventricle had not been entered yet, and the vector solution was then injected into the muscular wall. In some animals, a preparation was used in which the heart of a syngeneic animal was transplanted onto the abdominal aorta of a recipient littermate [17]. The heart could be kept alive in this fashion and could be injected by direct visualization of the apex of the myocardial wall, as previously described [17].

Percutaneous Infusions Into Porcine Coronary Vasculature

Access to the arterial system was obtained via cutdown to the right femoral artery. An 8F sheath was placed in the artery via the Seldinger technique. Systemic heparin (2,000 to 3,000 U) was administered. The left main coronary artery was engaged using an 8F hockey-stick guide catheter under fluoroscopic guidance. A Medtronic (Minneapolis, MN) transfer catheter was advanced into the mid-circumflex coronary artery over a 0.014-inch Hi-Torque (Advanced Cardiovascular Systems, Temecula, CA) floppy exchange length guidewire, which was then removed. One to 3 mL of an AAV stock containing 10^7 to 10^8 expressing units was then infused after verification of the catheter positions, followed by a flush with saline solution. During the entire study, 4 subjects were sacrificed 3 days after vector infusion, 2 subjects were sacrificed at 2 months, and 1 at 6 months after infusion. Two additional pigs served as sham controls for the study.

Tissue Preparation

All animals were cared for and sacrificed in accordance with the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals" (NIH publication 85-23, revised 1985). Rodents were sacrificed by intraperitoneal injection of a lethal dose of sodium pentobarbital followed by intracardiac perfusion of fixative. Pigs were sacrificed by overdose of intravenous sodium pentobarbital followed by excision of the heart and perfusion with fixative. Pig hearts were divided into several equally sized pieces for sectioning, with each piece numbered and the orientation within the original heart noted. Rat hearts were sectioned whole. Hearts were then incubated overnight in 30% sucrose/2 mmol/L MgCl₂/phosphate-buffered saline solution (PBS), pH 7.3, which serves as a cryoprotectant before sectioning. Thirty-micrometer sections were taken from fixed tissue using a freezing microtome, which were then processed for either β -galactosidase histochemistry or immunocytochemistry.

Histochemistry and Immunocytochemistry

Cells expressing enzymatically active β -galactosidase can be detected by incubation with the chromogenic substrate *X-gal*, which yields an insoluble blue precipitate when cleaved. For *X-gal* histochemistry, hearts were first perfused with a fixative that has previously been shown to permit *X-gal* staining of positive cells while completely eliminating background staining due to endogenous mammalian enzymes with β -galactosidase-like activity [16]. Sections were then permeabilized and incubated in *X-gal* substrate solution as described previously [16].

For β -galactosidase immunocytochemistry, tissue was fixed with 4% paraformaldehyde PBS, pH 7.3, by cardiac perfusion followed by overnight postfixation for 1 hour at 4°C. Tissue sections were taken as described above, then washed with PBS and blocked overnight with 10% goat serum in PBS at 4°C. The next day sections were treated rabbit polyclonal anti- β -galactosidase antibody (5'-3') at 1:500 dilution in PBS with 10% goat serum. After overnight incubation at 4°C and repeated PBS washes, the biotinylated goat anti-rabbit secondary antibody (Vector Labs, Burlingame, CA) in PBS with 10% goat serum was applied to sections for 1 to 2 hours at room temperature. Sections were again repeatedly washed with PBS. The ABC reagent consisting of avidin-biotin-horseradish peroxidase (Vector Labs) was prepared and applied to sections as per the manufacturer's instructions. After a final series of washes, bound horseradish peroxidase was detected by incubation for 3 to 5 minutes in the diaminobenzidine substrate solution described previously. Sections were then counterstained with hematoxylin and eosin to enable observation of the background tissue histology and architecture, and to permit detection of any infiltrating inflammatory cells.

Results

Gene Transfer Into Rat Heart Cells In Vitro and In Vivo Using an Adeno-Associated Virus Vector

AAVlac was first tested in primary culture to demonstrate that AAV vectors are capable of transferring genes into neonatal rat myocardial cells (data not shown). AAVlac was then infused into rat left ventricular myocardium via two methods. A transthoracic approach was first taken as described above. To obtain greater control over the localization of vector injections, we used a model in which a heart was transplanted onto the abdominal aorta of a syngeneic animal [17]. This heart maintains spontaneous contractile activity and normal cardiac histology for extended periods. This permitted full exposure of the left ventricle before injections without necessitating a respirator, which would be requisite for equivalent exposure of the functional heart. Cells positive for β -galactosidase histochemical activity were observed 3 days after injection by both methods, and animals sacrificed 2 months after injection also demonstrated positive cells (Fig 2B). No positive cells were observed in uninjected control subjects. At both time points positive cells were observed local to the injection site, with minimal spread. Furthermore, in the AAVlac-injected subjects the surrounding tissue appeared to be healthy, with no evidence of significant inflammation or damage as a result of viral transduction.

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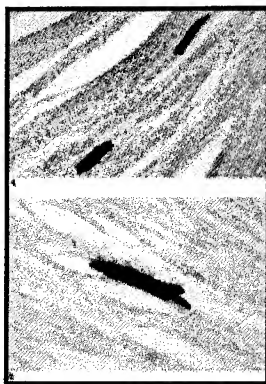


Fig 2. . Positive rat cardiac muscle cell after histochemical assay for β -galactosidase activity. This assay results in a blue color change in cells expressing the enzyme β -galactosidase. These figures were taken from animals sacrificed 3 days (A) and 2 months (B) after direct, intramuscular injection of AAVlac into the myocardium of an adult rat. ($\times 20$ before 44% reduction.)

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Gene Transfer Into Pig Myocardium In Vivo Via Percutaneous, Fluoroscopically Guided Catheter Infusion of Adeno-Associated Virus Vectors Into the Coronary Vasculature

To determine whether AAV vectors can transfer genes into myocardial cells in a more clinically relevant model, AAVlac was infused into the coronary arteries of adult pigs. A percutaneously introduced infusion catheter was guided to the coronary ostium under fluoroscopy. Contrast dye was then injected to demonstrate the location of the catheter tip. In all subjects, the catheter tip was guided to the **circumflex** coronary artery (Fig 3A). All subjects thus received infusions into the same vessel.



Fig 3. . Placement of the infusion catheter into the **circumflex** coronary artery. This is an image of the catheter under fluoroscopy after guidance to the coronary arteries. When the tip was believed to be at the opening of the **circumflex** artery (left; arrow), contrast dye was infused (right). The contrast dye can be seen enhancing only the **circumflex** coronary artery and its branches, which feeds the posterolateral wall of the left ventricle. The tip (arrow) was thereby determined to be correctly placed, and after flushing with saline solution, the adeno-associated virus vector was infused.

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Purified AAVlac infusion into the porcine **circumflex** coronary artery resulted in expression of the β -galactosidase reporter gene in cardiac muscle cells. At 3 days after infusion, animals were sacrificed and tissue sections were processed for immunocytochemical detection of β -galactosidase. This assay was used for the porcine studies due to greater sensitivity in demonstrating completely filled muscle cells as well as the ability to use a stronger fixative, which permitted easier manipulation of large numbers of tissue sections. Numerous positive cells were observed across a large area within the distribution of the **circumflex** coronary artery (Fig 4A, B). No positive cells were observed in control sections from animals infused with saline solution (4F); positive cells were also absent from regions not supplied by the **circumflex** artery in AAVlac-treated subjects.

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Fig 4. Immunocytochemical detection of porcine heart cells expressing β -galactosidase. (A, B) Positive myocardial cells 3 days after infusion of AAVlac. Note the elongated, muscular morphology of most of the positive cells. Section A and B were from the same animal but were separated by 450 sections. (C) Positive cells from an animal sacrificed 2 months after infusion of AAVlac. The morphology of the cells at 2 months was of slightly poor quality due to cutting artifact when these sections were taken. (D, E) Positive cells from the heart of a pig sacrificed 6 months after infusion of AAVlac into the circumflex coronary artery. Sections D and E were from the same animal but were separated by 240 sections. (F) A section of myocardium from a mock-infused control subject. This section was stained with substrate longer than all other sections to ensure the complete lack of positive staining. Although this resulted in a greater edge artifact seen as a slight increase in overall brown tone, there are clearly no genuine positive brown cells. Note the absence of any inflammatory cells or tissue damage (other than cutting artifact) in any of the tissue samples. (x10 before % reduction.)

Although most positive sections contained no more than 200 immunoreactive cells, positive cells were noted in a large number of sections within the area perfused by the circumflex artery. For example, Figure 4A shows cells from a section that contained a total of 156 positive cells. Figure 4B demonstrates cells from a section in the same animal that contained a total of 87 positive cells. These represented section 17 and section 2, respectively, from the original tissue block. Because every 30th section was taken, these two sections were thus separated by 450 30- μ m sections, or a distance of 13.5 mm. Because each intervening section demonstrated on average 100 cells or more, the estimated number of positive cells in this block would be 4.5×10^4 . This does not represent all positive cells from this block because smaller numbers of positive cells were observed in sections at least 250 sections removed from section 17. In addition, numerous positive cells were also observed in a second adjacent tissue block perfused in part by the circumflex coronary artery, although fewer positive sections were observed. Thus the number of positive cells is estimated to be 10^5 . Because the titer of virus used was approximately 5×10^7 units/mL and 1 mL was infused into each subject, the efficiency of gene transfer is estimated to be roughly 0.2% assuming that these data account for all potential positive cells and that each positive cell represents gene transfer by 1 vector particle.

Pigs sacrificed 2 months and 6 months after infusion also demonstrated positive cells (Figs 4C–E, 5A–C). Numerous fully labeled muscle cells were again observed largely limited to the distribution of the circumflex coronary artery. The number of positive cells seen at 2 months after infusion appears to have decreased to approximately 25% of the number seen at 3 days after infusion. By 6 months the number of cells appears to have stabilized and was roughly equivalent to the number of immunoreactive cells observed at 2 months after infusion.



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Fig 5. High-power view of two cells expressing β -galactosidase 6 months after infusion of AAVlac. Note the presence of the diaminobenzidine brown product within the borders of two individual muscle cells. This photomicrograph was taken with slight phase contrast, and intercalated discs can be appreciated upon close observation. Note the complete lack of inflammatory cells surrounding the positive muscle cells. (x40 before 46% reduction.)

The distribution of positive cells at 6 months appears to be equivalent to the distribution at 3 days after infusion. As an example, Figure 4D shows cells from section 12 of the 6-month animal, which contained a total of 43 positive cells, whereas Figure 4E was taken from section 4, which totaled 39 positive cells. Again each numbered section was separated by 30 sections, so these two slides were actually 240 sections apart. As in the 3-day animals, there was no evidence of toxicity or inflammation due to AAV-

mediated gene transfer at any time point. Figure 5² demonstrates a high-powered view of two immunoreactive cardiac muscle cells. This clearly demonstrates both the presence of intracellular reaction product and a complete lack of inflammatory cells around the positive muscle cells.

Comment

This report demonstrates that AAV vectors can transfer genes into the mammalian heart. Previously adenoviral vectors have been used for gene transfer into the adult myocardium. As with any recombinant vector, adenovirus can easily be grown to very high titers because vector reproduction occurs simply by viral replication. Thus, a large number of viral particles can be injected in a small volume. By contrast, AAV vector reproduction requires expression of proteins from the adenovirus genome, which then promote expression of AAV genes from a distinct plasmid. The resulting AAV proteins must then efficiently replicate and package the vector DNA from yet another plasmid. As a result AAV vectors do not grow to the same titers as adenovirus. Although we observed positive muscle cells at both 3 days and 2 months in rodent heart after direct needle injection of AAV vectors, it is clear that far fewer cells were observed in this study compared with prior reports using adenoviral vectors in rodents [3, 4].

However, these studies also used adenoviral stocks that were several orders of magnitude more concentrated than the stock used in our study. Thus, AAV-mediated myocardial gene transfer appears to be at least as efficient as adenoviral transduction of heart cells. This is consistent with prior studies that have documented highly efficient AAV-mediated gene transfer in other organ systems [12–15]. A more concentrated AAV stock was used for the porcine studies, and a far greater number of positive cells was also observed. The porcine data cannot readily be compared with prior reports of adenovirus gene transfer because we used a very different approach to gene delivery in the pig heart. However, if the number of positive cells is compared with the number of input vector particles, then it is clear that the AAV vector is at least as efficient at short-term delivery as other systems. Adeno-associated virus vectors can now achieve titers of greater than 10^9 particles/mL in proficient hands, which is comparable with adenovirus titers that have been used to positively alter physiology in other organ systems. This suggests that physiologically meaningful numbers of positive cells can be achieved in the myocardium using the AAV approach.

Long-term expression of the bacterial *lacZ* gene was observed in pig hearts transduced with the AAV vector. Although gene transfer with adenoviral vectors has resulted in large numbers of positive cells for up to 7 to 10 days, expression has generally declined with time and appears to cease at 3 to 4 weeks after vector infusion [3–7]. In the current study, expression was observed to at least 6 months after AAV transduction of pig myocardium. Because relatively few positive cells were observed in rat hearts at short time points, it was not surprising that a small number of cells expressed *lacZ* at 2 months. In the pig heart, however, a large number of positive cells were observed at 2 and 6 months after AAV infusion, consistent with the larger number of positive cells seen at 3 days. The slight decline in the number of positive cells observed at longer time points is consistent with earlier observations in other organs. On a percentage basis, however, our data demonstrate that AAV yields stable expression in pig myocardium for at least 6 months after vector infusion.

Another feature of the current study was the absence of apparent inflammatory response to AAV transduction in the mammalian heart. Inflammation has been invoked as a possible explanation for the limited longevity of expression after adenoviral gene transfer into myocardial cells. Immune cell infiltrates have been observed surrounding adenovirus-transduced myocardial cells [6]. This is likely due to the presence of viral genes within the replication-incompetent vectors that continue to express adenovirus gene products within target cells. These products are then presented to the immune system, which recognizes them as foreign and reacts to the cell as if it were infected with a wild-type virus (see Fig 1²). The resulting immune reaction may not only result in tissue damage, but also could play a role in limiting the length of gene expression either by inducing target cell degradation of vector DNA or by destruction of the transduced cell. Because the AAV vector does not contain any viral genes, the potential for immune reactions against transduced cells due to synthesis of viral proteins is eliminated (see Fig 1²). This not only results in a vector with increased safety, but may actually improve longevity of gene expression.

In addition to the unusual vector used for this study, we used a different approach for delivery of viral vectors to the heart of a large mammal. Percutaneous infusion of fluids into the coronary vasculature via fluoroscopically guided cardiac catheterization is a routine procedure. Because this technique usually does not require hospital admission, it is attractive as a safe, simple, and cost-

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effective method for cardiac gene delivery. Furthermore, if a viral vector can penetrate effectively into the myocardium, this also presents the possibility of widespread gene delivery throughout the heart.

Previous studies in the rodent and porcine heart have used direct needle injection of adenovirus into a point within the left ventricular wall [3–6]. A percutaneous approach has been used in one canine study; however, the end of the catheter contained a needle that was floated into the lumen of the left ventricle and inserted into the myocardium [7]. Therefore, this approach also resulted in direct intramyocardial vector injection. Adenovirus has been infused into the rabbit coronary vasculature using a percutaneous approach [5]. Our data in a large, clinically relevant animal indicate that AAV vectors can penetrate into the myocardium to yield widespread gene delivery limited to the left ventricular region perfused by the injected artery.

Adeno-associated virus is presented here as an approach to in vivo gene transfer in the adult myocardium. The ability to genetically modify large numbers of myocardial cells in a safe and stable fashion suggests that this may have significant clinical utility for gene therapy of diseases previously refractory to conventional treatments. The use of a common, endovascular catheterization technique for introduction of vectors to a selective region of the myocardium further enhances the potential of this approach for application in a variety of clinical settings.

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Doctor Xiao, who assisted in this work while in Dr Samulski's laboratory, works for Somatix Therapy Corporation, which owns the rights to commercialize AAV vector therapy. Somatix Therapy Corporation provided no funding for or assistance with this project.

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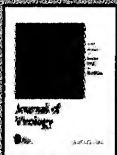
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EXHIBIT G



Lipid-mediated gene transfer of viral IL-10 prolongs vascularized cardiac allograft survival by inhibiting donor-specific cellular and humoral immune responses

LA DeBruyne, K Li, SY Chan, L Qin, DK Bishop and JS Bromberg

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The gene encoding the immunosuppressive cytokine viral interleukin-10 (vIL-10) was introduced into BALB/c (H-2^d) vascularized cardiac allografts by perfusing the graft vasculature with DNA-liposome complexes, utilizing the experimental cationic lipid γ -AP DLRIE/DOPE and a plasmid encoding vIL-10 under the control of the HCMV promoter. The DNA to lipid ratio and DNA dose were critical factors in obtaining optimal biologic effects. Gene transfer of vIL-10 with a 3:1 DNA to lipid weight ratio using 375 μ g DNA significantly prolonged allograft survival in MHC-mismatched C57BL/6 (H-2^b) recipients (16.00 days) compared with both unmodified allografts (8.14 days) and vIL-10 antisense controls (6.28 days). Enhanced graft survival was specific to vIL-10 expression since treatment with antisense plasmid or anti-vIL-10 monoclonal antibody (mAb) abrogated the effect. Prolonged survival was associated with a novel histology characterized by a moderate mono-

nuclear infiltrate, edema, and diffuse fibrillar/collagen deposition in the interstitium. Despite these morphologic changes, myocytes remained viable and vessels were patent. Limiting dilution analysis revealed transient infiltration of IL-2 secreting, donor-reactive, helper T lymphocytes (HTL) and cytotoxic T lymphocytes (CTL) in vIL-10 expressing grafts on day 7, that decreased significantly by day 14. Similarly, vIL-10 gene transfer inhibited the accumulation of donor-specific HTL and CTL in the spleen, compared with antisense controls. Prolonged survival was also associated with a marked decrease in IgM and IgG alloantibody production, with little to no IgG isotype switching. These results show that viral IL-10 gene transfer inhibits graft rejection in a clinically relevant model by inhibiting donor-specific cellular and humoral immune responses.

Keywords: viral interleukin-10 (vIL-10); gene transfer; transplantation; immunotherapy

Introduction

Organ transplantation is an effective therapy for many end stage diseases. A major complication associated with transplantation is allograft rejection which is controlled by administering immunosuppressive pharmacologic agents to allograft recipients; however, systemic immunosuppression often leads to opportunistic infections and drug-specific side-effects. Thus, there exists a great need to develop more specific immunosuppressive therapies. This goal may be achieved by delivering immunomodulatory signals specifically to the regions of antigen presentation. Gene transfer has the potential to introduce immunoregulatory molecules directly into the graft, producing a localized immunosuppressive environment without systemic side-effects.^{1,2}

The major factor limiting the success of gene therapy is that transferred genes are expressed only transiently and at low levels in only a small number of target cells.³ However, these factors may not be deleterious in transplantation applications.⁴ The transient expression of a gene following graft placement may provide the appropriate signals to prevent the initiation of an immune

response and induce tolerance. In addition, transfection of only a small number of cells may be efficient when transferring genes encoding soluble, secreted immunosuppressive molecules.

IL-10 is an important regulatory molecule that possesses immunosuppressive activity and was originally identified by its ability to inhibit cytokine synthesis of Th1 cells, which produce IFN- γ and IL-2 and mediate DTH responses and CTL development.⁵ IL-10 is predominately produced by macrophages and Th2 cells. Th2 cells produce IL-4, IL-5 and IL-10 which promote humoral, mucosal and allergic responses.⁶ Th1 and Th2 cells are antagonistic such that IFN- γ promotes Th1 development and inhibits Th2 proliferation. In contrast, IL-10 inhibits cytokine production by Th1 cells but not Th2 cells.⁷ It has been proposed that IL-10 mediates this activity by inhibiting IL-12 production, a key regulatory cytokine in promoting Th1 development.^{8,9} It should be noted that IL-10 also inhibits the production of monokines, including IL-1, IL-6, IL-8, TNF- α , GM-CSF and G-CSF.^{10,11} In addition to inhibiting cytokine production, IL-10 has also been shown to block accessory cell function of macrophages by down-regulating MHC class II expression and decreasing expression of the costimulatory molecule B7.^{12–15} As a result of these activities, IL-10 is a potent suppressor of effector functions of macrophages and T cells. However, in addition to regulating immune

responses negatively, IL-10 also has growth factor activity and promotes the proliferation and differentiation of B cells, mast cells and thymocytes.¹⁶⁻¹⁸ A homologue of IL-10 encoded in the Epstein-Barr *BCRF1* open reading frame, designated viral IL-10, has activity on both human and mouse cells.¹⁹ Viral IL-10 shares many of the biologic activities of cellular IL-10, including inhibition of cytokine synthesis and accessory cell function.^{5,10,12} However, vIL-10 does not possess the T cell costimulatory activities of cellular IL-10.²⁰⁻²² Thus, the introduction of vIL-10 into allografts by direct gene transfer should reduce immunogenicity, impair effective antigen presentation, and suppress macrophage and T cell effector function, thereby inhibiting graft rejection.

Previous studies from our laboratory showed that a variety of plasmid and viral (retrovirus, herpesvirus, adenovirus) vectors can successfully transfer and express exogenous genes in cardiac allografts.^{1,2,23,24} The duration and distribution of transferred gene expression depended on vector characteristics. These studies showed that the transient, local expression of the gene encoding vIL-10 can generate local immunosuppression by inhibiting donor-specific HTL and CTL, making gene transfer a therapeutic option for preventing allograft rejection. These previous studies utilized the nonvascularized, neonatal heart, ear pinna model. Although informative, this model system is not directly clinically applicable. Thus, the present study addresses the plausibility of utilizing vIL-10 gene transfer in a clinically relevant vascularized model.

The development of effective vectors for vascular gene delivery is an absolute requisite if gene therapy approaches are to be successful in solid organ transplantation. Although adenoviral vectors have been shown to be effective for such purposes in animal models, the ability to achieve comparable gene transfer efficiency with nonviral vectors would be safer, less toxic, and more applicable to humans. A new cationic liposome preparation, designated γ AP-DLRIE/DOPE, is an efficient liposomal vector that has recently been shown to increase *in vivo* vascular gene expression by greater than 15-fold compared with naked DNA or previously described cationic lipids.²⁵ These levels of gene expression represent a significant improvement in nonviral vector *in vivo* transfection efficiency and approach levels observed with clinically acceptable doses of adenoviral vectors.²⁵ Thus, the present study utilizes γ AP-DLRIE/DOPE to deliver *in situ* a vIL-10 encoding plasmid to the vasculature of donor cardiac allografts and identifies immunologic mechanisms associated with enhanced graft survival.

Results

vIL-10 gene transfer and expression in cardiac allografts
BALB/c cardiac allografts were perfused with different doses of plasmid DNA encoding vIL-10 complexed with γ AP DLRIE/DOPE, and graft survival was determined following transplantation into C57BL/6 recipient mice (Table 1). The total amount of plasmid DNA utilized and the ratio of DNA to lipid both influenced graft survival. High lipid concentrations ($>600 \mu\text{g/ml}$) resulted in vascular hemorrhage, which may have been an indication of lipid toxicity. As shown in Table 1, graft survival improved as the total amount of DNA and the DNA:lipid

Table 1. Effect of DNA and lipid concentration on graft survival

DNA:Lipid ($\mu\text{g}:\mu\text{g}$)	DNA ($\mu\text{g/ml}$)	Lipid ($\mu\text{g/ml}$)	Mean graft survival (days)
0.5	300	600	7.0
0.5	100	200	8.0
1.0	300	300	9.0
2.0	600	300	9.0
2.0	1000	500	9.0
3.0	900	300	11.0
3.0	1500	500	16.0

BALB/c cardiac allografts were perfused with different doses of plasmid DNA encoding vIL-10 complexed with γ AP DLRIE/DOPE, and graft survival determined following transplantation into C57BL/6 recipient mice. Optimal gene transfer (as measured by enhanced graft survival) was demonstrated at a dose of 1500 $\mu\text{g/ml}$ DNA combined with 500 $\mu\text{g/ml}$ lipid, which significantly prolonged graft survival compared with controls (16.0 days versus 8.0 days, $P=0.02$).

charge ratio increased. For these studies, optimal gene transfer (as measured by enhanced graft survival) was demonstrated at a dose of 1500 $\mu\text{g/ml}$ DNA combined with 500 $\mu\text{g/ml}$ lipid, which significantly prolonged graft survival compared with controls (16.0 days versus 8.14 days, $P=0.02$) and was used in the remainder of these studies. It was not possible to increase further the DNA:lipid ratio, since a ratio of 4.0 at high DNA-lipid concentrations resulted in precipitation of the complex. As a control for DNA-lipid effects, donor cardiac allografts were perfused with DNA-liposome complexes encoding vIL-10 in the antisense orientation. As shown in Table 2, there was no significant difference in graft survival between unmodified allografts and antisense controls ($P=0.72$). In addition, animals receiving vIL-10-transduced grafts treated with an anti-vIL-10 mAb, which does not cross-react with cellular murine IL-10, rejected their grafts similarly to controls, demonstrating that expression of the vIL-10 transgene was required for prolonged graft survival.

To address the question of intragraft transgene expression, RT-PCR analysis was performed on the donor hearts of animals receiving vIL-10-transduced grafts. Animals were killed and donor heart tissue analyzed by RT-PCR on days 1, 7 and 14 while the hearts were still functioning, as indicated by abdominal palpation. All hearts evaluated expressed the vIL-10 transgene (Figure 1). Although these PCR assays were not quantitative, expression appeared strongest early on (day 1) and was harder to detect further out (day 14). Since donor allografts perfused with vIL-10 DNA-liposome complexes were not flushed clear of vector before anastomosis of the graft into the recipient, it was likely that some vIL-10 plasmid DNA localized to other anatomic regions in addition to the graft. It was therefore important to determine if vector DNA outside the graft contributed to graft survival and immunosuppression by gene expression. RT-PCR analysis on liver, lung, kidney, spleen, mesenteric lymph nodes, and native heart from animals receiving vIL-10-transduced grafts detected vIL-10 expression in all tissues at 7 days after transplantation, albeit at low levels in most samples (Figure 2). However, by 14 days

Table 2 Lipid-mediated gene transfer of vIL-10 prolongs graft survival

Treatment	Graft survival (days)	Mean \pm S.E. (days)	P
vIL-10 sense	3, 12, 12, 14, 14*, 14*, 14*, 14*, 14*, 16, 20, 40	16.00 \pm 2.32	0.021
vIL-10 antisense	7, 8, 8, 8, 9, 9, 9	8.28 \pm 0.29	0.718
Unmodified control	7, 8, 8, 8, 9, 9, 9	8.74 \pm 0.25	—
vIL-10 sense + anti-vIL-10 mAb	7, 7, 9	7.67 \pm 0.66	0.430

All donor grafts were perfused with 250 μ l of the optimal DNA-lipid complex (1500 μ g/ml DNA/500 μ g/ml lipid).

*Five animals were killed on day 14 with functioning grafts which were used for immunologic analysis. These animals were included in the calculation of the mean survival and given a day 14 value, resulting in a mean of greater than 16 days.



Figure 1 Viral IL-10 transgene transcription in cardiac allografts. RT-PCR was performed on DNase-treated RNA isolated from functioning grafts on days 1, 7 and 14 after transplantation. Lane 1, positive control (PCR using pMP6A vIL-10 as template); lane 2, negative control (PCR without cDNA template); lane 3, RT-PCR on vIL-10-transduced allograft on day 1; lane 4, RNA-PCR without RT enzyme on day 1 graft (negative control for DNase activity); lane 5, RT-PCR on day 7 vIL-10 graft; lane 6 RNA-PCR without RT enzyme on day 7 graft; lane 7, RT-PCR on day 14 vIL-10-transduced graft; lane 8, RNA-PCR without RT enzyme on day 14 graft.

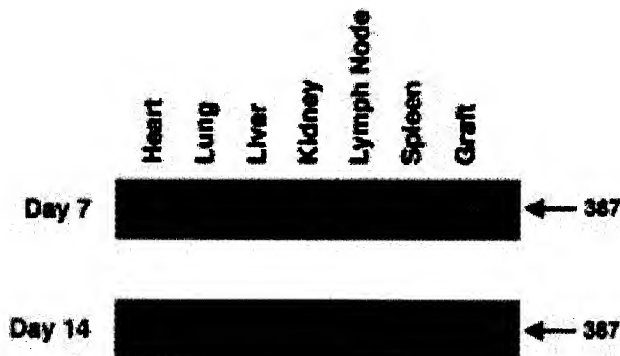


Figure 2 Viral IL-10 gene transcription is detectable in tissues other than the allograft. RT-PCR was performed on DNase-treated RNA isolated from native heart, lung, liver, kidney, mesenteric lymph node, spleen and allograft on days 7 and 14 after transplantation. Viral IL-10 primers generate a PCR product of 387 base pairs.

after transplantation vIL-10 gene transcription was only detectable in the allograft. Greater gene transfection might be expected within the graft since the allograft remained in contact with the DNA-lipid complexes for an extended time-period under static conditions, whereas upon anastomosis the remaining plasmid DNA was flushed out of the allograft and into the bloodstream.

Viral IL-10 gene transfer decreases intra-graft and systemic donor-specific cell-mediated immunity

Limiting dilution analysis was performed on graft infiltrating cells and splenocytes to investigate the immunologic effects of vIL-10 gene transfer. A modification of

this assay was used that allows for the differentiation between the total number of precursor donor-reactive T lymphocytes (tHTL and tCTL) and *in vivo* activated or 'conditioned' donor-reactive T lymphocytes (cHTL and cCTL). These analyses were performed at least three times on vIL-10 sense and antisense control animals on days 7 and 14 after transplantation. It should be noted that it was not possible to assess graft infiltrating HTL and CTL in antisense control grafts on day 14, since by that point the grafts which had rejected on days 8 or 9 had become masses of necrotic tissue that did not yield appreciable numbers of graft infiltrating cells for immunologic analysis.

Limiting dilution analysis of graft infiltrating cells on day 7 after transplant revealed no difference in the total number of donor-specific graft infiltrating tHTL or cHTL compared with antisense controls (Figure 3a). However, by day 14 after transplant, there was a significant decrease in the number of both donor-specific cHTL and tHTL infiltrating the graft in vIL-10-transduced hearts compared with day 7 sense and antisense controls. This decrease in HTL numbers was detectable in three of three experiments. The observed local decrease in graft infiltrating HTL in vIL-10-transduced recipients correlated with a reduction in donor-specific splenic HTL (Figure 3c). On day 7 after transplant, the number of donor-specific splenic HTL in vIL-10-transduced recipients was similar to antisense controls, although by day 14 these numbers dropped significantly in the experimental group. In contrast, the number of splenic cHTL and tHTL in antisense controls remained constant between days 7 and 14, as has been previously reported in animals receiving unmodified allografts.²⁶ In summary, vIL-10 gene transfer resulted in a transient infiltration of donor-specific HTL in the allograft which subsided by day 14 after transplant, correlating with a decrease of donor-specific HTL in the spleen.

Analysis of graft infiltrating cytolytic cells on day 7 revealed that fewer donor-specific cCTL and tCTL infiltrated vIL-10-transduced grafts compared with controls, and that this decrease persisted on day 14 (Figure 3b). Although this decrease was small (2.6-fold) it was reproducible in three of three experiments. Limiting dilution analysis of splenocytes from antisense controls showed a marked expansion of tCTL on day 7 after transplant (Figure 3d), which also normally occurs in recipients of unmodified allografts.²⁶ This expansion of donor-specific tCTL was not present in animals receiving vIL-10-transduced grafts. The expansion of splenic tCTL in antisense controls subsided by day 14 after transplant, as has been

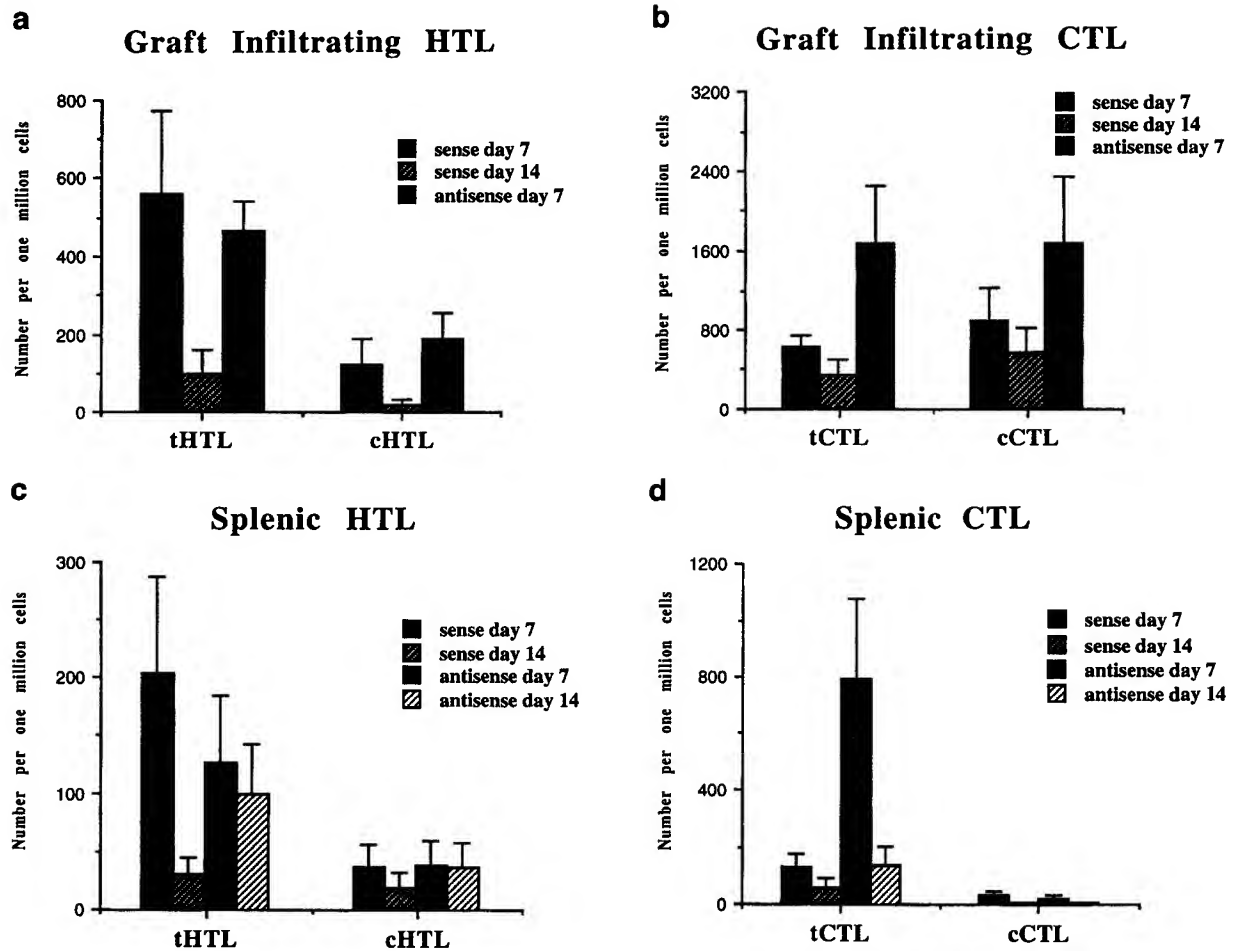


Figure 3 Limiting dilution analysis of donor-specific cells. A modification of this assay was used that allows for the differentiation between the total number of precursor donor-reactive T lymphocytes (tHTL and tCTL) from those that have been activated or 'conditioned' in vivo (cHTL and cCTL). Results are presented as number per one million cells, including the 95% confidence interval. (a) Graft infiltrating donor-specific HTL; (b) graft infiltrating donor-specific CTL; (c) splenic donor-specific HTL; (d) splenic donor-specific CTL.

reported in recipients of unmodified allografts.²⁶ Activated splenic CTL were rare in both controls and in animals with vIL-10-transduced grafts. Thus, these findings reveal that vIL-10 gene transfer results in a modest decrease in graft infiltrating cCTL and tCTL, and inhibits splenic tCTL expansion.

Viral IL-10 gene transfer is associated with decreased alloantibody production

The decrease in the number of graft infiltrating helper T cells suggested that there might be a concomitant decrease in donor-specific alloantibody production (Table 3). The results show that animals receiving vIL-10 perfused donor hearts produced significantly less IgM alloantibody at days 7 and 14 compared with both antisense and unmodified controls. In fact, significant levels of donor-specific IgM alloantibody were only detectable in serum samples from mice receiving suboptimal vIL-10-transduced grafts that did not exhibit prolonged survival. Animals that received donor grafts transduced with the optimal DNA and lipid doses and then treated with an anti-vIL-10 mAb also produced high levels of IgM allo-

Treatment	Day	IgM	IgG1	IgG2a
vIL-10 sense	7	10.41	2.37	3.29
	14	0.30	3.57	3.42
vIL-10 antisense	7	18.77	6.78	4.99
	14	18.30	65.91	65.59
Unmodified control	7	41.18	8.49	7.23
	14	27.42	45.99	45.02
vIL-10 sense + anti-vIL-10 mAb	7	36.27	9.36	9.04
0.5 DNA:lipid ratio	8	57.75	12.60	13.74
1.0 DNA:lipid ratio	9	49.84	12.63	10.24
2.0 DNA:lipid ratio	9	56.55	15.79	19.85
Isotype controls		2.99	2.92	2.82

Alloantibody results are presented as mean channel fluorescence. All groups were treated with the optimal 3.0 DNA:lipid ratio of vIL-10 sense plasmid to γAP DLRIE/DOPE, unless otherwise indicated.

antibody, further indicating that vIL-10 is directly responsible for inhibiting donor-specific IgM alloantibody production.

One potential mechanism by which vIL-10 gene transfer may induce prolonged graft survival is by inducing a Th2-type immune response. Differential production of Th1 and Th2 cytokines can be reflected in alloantibody isotype production. IgG1 is indicative of a Th2-type response, whereas IgG2a is indicative of a Th1-type immune response. In these studies, little to no IgG of either isotype was detectable in animals receiving donor grafts transduced with the optimal DNA-lipid dose. However, large amounts of both IgG1 and IgG2a were detected in both antisense and unmodified controls, as well as in animals receiving suboptimal DNA and lipid doses or in animals treated with anti-vIL-10 mAb. In the animals that exhibited IgG production, there was no dominant isotype produced; all animals exhibited comparable amounts of IgG1 and IgG2a.

vIL-10 gene transfer is associated with fibrosis of cardiac allografts

Donor syngeneic and allogeneic cardiac grafts were fixed in formalin, sectioned, and stained with either hematoxylin and eosin or trichrome for examination by light microscopy (Figure 4). Prolonged survival of vIL-10-transduced allografts was associated with a novel histology characterized by a moderate mononuclear infiltrate composed largely of unactivated cells, as distinguished by their low cytoplasm to nucleus ratio, marked edema, and diffuse collagen deposition in the interstitium (as indicated by trichrome staining); yet myocytes remained viable and vessels were patent (Figure 4e and f). Since it has been reported that cellular IL-10 up-regulates elastin gene expression *in vivo*, elastin stains were also performed, but there was no indication of abnormal elastin production (data not shown).²⁷ These manifestations did not occur in syngeneic grafts perfused with the vIL-10 DNA-liposome complexes (Figure 4g and h), implying that the observed fibrosis was not a direct effect of the vIL-10 gene product, lipid-associated toxicity, or an artifact of the perfusion protocol. In addition, there was no evidence of fibrosis in allografts perfused with antisense vIL-10 DNA-liposome complexes (Figure 4c and d). These findings suggest that both the vIL-10 transgene product and an alloantigenic component are required for the observed effect.

Discussion

This study addressed the feasibility of utilizing vIL-10 gene transfer as a therapeutic modality for inhibiting cardiac allograft rejection in a clinically relevant model and addressed the immunomodulatory mechanisms associated with enhanced graft survival. Lipid-mediated gene transfer of vIL-10 was shown to significantly prolong allograft survival without conventional immunosuppression and the effect was specific in that antisense constructs or treatment with an anti-vIL-10 mAb negated the effect. The present study did not examine the specific cell types transduced or the precise location of transgene expression. However, previous *in vivo* studies of lipid-mediated gene transfer to pig vasculature using lipofectin or γ AP DLRIE/DOPE demonstrated that transfection was site limited and resulted in reporter gene expression

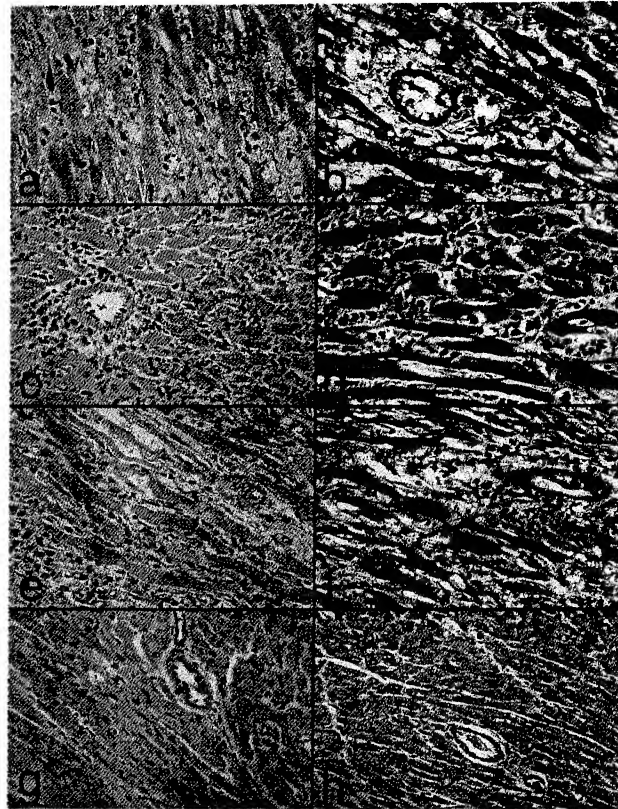


Figure 4 Prolonged survival of vIL-10 transduced allografts was associated with a novel histology characterized by a moderate and quiescent mononuclear infiltrate, marked edema, and diffuse collagen deposition throughout the interstitium. Grafts were sectioned and stained with either hematoxylin and eosin (a, c, e and g) or trichrome (b, d, f and h) for examination by light microscopy ($\times 400$). Collagen deposition (bright blue) is visible in trichrome-stained tissue sections. (a) and (b), vIL-10 sense allografts at day 7 after transplant; (c) and (d), vIL-10 antisense allografts at day 7 after transplant; (e) and (f), vIL-10 sense allografts at day 14 after transplant; (g) and (h), vIL-10 sense syngeneic grafts at day 14 after transplant.

in vessel endothelial cells and smooth muscle cells.^{25,28} This is in agreement with reports using lipofectin in the vascularized mouse cardiac transplant model.^{29,30} Using a luciferase transgene, investigators reported that perfusion of DNA-liposome complexes via the coronary arteries resulted in expression of the transgene primarily in the coronary capillaries, in perivascular areas surrounding coronary arteries and veins, and the endocardia of both ventricles.

Other studies using adenoviral vectors have perfused cardiac allografts and isografts and shown similar reporter results, except that adenoviral transgene expression was not confined to the coronary vasculature (including endothelial cells and smooth muscle cells of the vessels), but was also present in the myocytes.³¹⁻³⁴ The higher efficiency of viral vectors at delivering genes *in vivo* than nonviral vectors is illustrated by a direct comparison in which luciferase transgene activity was 10-fold higher in rat cardiac grafts transduced with an adenoviral vector compared with a plasmid vector.³⁵ None the less, even with viral vectors, investigators have documented inefficient gene transfer by allograft perfusion, with most studies reporting less than 1% of the cells expressing the

transgene.^{30,33} Although appealing because they can transduce a wide variety of cell types relatively efficiently, adenoviral vectors induce an exuberant immune response and can be especially problematic in human gene therapy applications, in that many individuals have pre-existing immunity to adenovirus.³ Our laboratory recently demonstrated that the vIL-10 transgene can inhibit not only alloantigenic responses, but also anti-adenoviral immune responses.³⁶ The results showed that *in vivo* treatment with a vIL-10 expressing adenoviral vector prevented adenovirus-specific CTL priming and generation. Not only did the vIL-10 gene product inhibit the immune response toward adenoviral antigens, it improved the persistence of the vector and extended transgene expression. Thus, the efficacy of viral vectors may be further improved by incorporating immunosuppressive genes, such as vIL-10, into the vector.

Although vIL-10 gene transfer prolonged graft viability, survival was not indefinite and tolerance was not achieved. This was likely due to the fact that lipid-mediated plasmid gene transfer was inefficient and expression was low and transient. Even though viral vector gene transfer may be more efficient than plasmid-based vectors, plasmids are likely to be safer and more clinically applicable to transplantation and thus deserving of continued testing and scrutiny. Additional approaches will be required to improve gene transfer and expression such as optimization of nucleic acid delivery, transcellular transport, nuclear localizing signals and selective promoters.^{1,3,4,25}

We previously demonstrated that a number of plasmid and viral vectors could successfully transfer and express vIL-10 in cardiac transplants.^{1,2} These past studies utilized the neonatal heart, ear pinna model. In this nonvascularized model, retroviral vectors encoding vIL-10 were found to prolong significantly allograft survival and inhibit donor-specific CTL and HTL as well as CD4⁺ and CD8⁺ lymphocytic infiltration within the graft.² In the present study, which uses a vascularized cardiac allograft model, we detected only a slight decrease in the number of donor-specific CTL, but did find a marked decrease in donor-specific intragraft and splenic IL-2 producing HTL. The large decrease demonstrated in both intragraft cHTL and tHTL at day 14 after transplant might suggest a decrease in the absolute number of T cells present, as a result of deletion or anergy. An alternative explanation for this finding is that since vIL-10 is known to induce Th2 cells, donor-specific HTL may have had a change of function, now producing IL-4 instead of IL-2 in response to donor alloantigen. These separate mechanisms are not necessarily mutually exclusive. Limiting dilution analysis of splenocytes from these animals indicated that there was also a detectable decrease in the number of donor-specific tHTL from 1/4849 on day 7 to 1/33 573 on day 14 after transplantation in the spleens of these animals. This demonstrates not only local, intragraft immunosuppression, but also a systemic immunosuppressive effect as well, and makes it unlikely that the decrease in intragraft HTL was due to egress of HTL from the graft into the systemic lymphocyte pool. In addition, the fact that vIL-10 gene transfer inhibited splenic expansion of donor-specific tCTL further suggests systemic, donor-specific immunosuppression. Future studies will focus on differentiating the mechanisms responsible for decreased

donor-specific HTL and CTL numbers, and their influence on graft survival.

Mechanistically, vIL-10 gene transfer resulted in decreased alloantibody production with little to no IgG class switching. Viral IL-10 is a known growth factor for B cells and has been shown to increase antibody production *in vivo*.¹⁷ Thus, it is unclear why vIL-10 gene transfer resulted in inhibition of alloantibody production. Possibly other biologic effects of vIL-10, such as blocking accessory cell function by down-regulating MHC class II expression and decreasing expression of the costimulatory molecule B7, impair effective antigen presentation and T cell helper function. None the less, it is clear from findings in this study that vIL-10 gene transfer can inhibit both humoral and cellular components of the immune response.

Our findings are in contrast to reports that expression of an IL-10 transgene in pancreatic islets did not inhibit allograft rejection or autoimmunity.^{21,37,38} However, these studies utilized cellular IL-10 which possesses immunostimulatory activities, including enhancing MHC class II expression and inducing cytotoxic T cell differentiation, that are not properties of vIL-10. In addition, recent studies have shown that both vIL-10-transduced islets and hepatic allografts produce biologically relevant amounts of vIL-10 that inhibit alloantigenic lymphocyte proliferation *in vitro*.^{39,40} Furthermore, it was reported that mice receiving vIL-10-transduced allogeneic tumors develop local anergy to the tumors, whereas mice receiving the same allogeneic tumors transduced with cellular IL-10, suppress tumor growth and frequently reject the tumor.²² Finally, a direct comparison in our laboratory utilizing retroviral vectors encoding either vIL-10 or cellular IL-10 demonstrated that treatment of nonvascularized cardiac allografts with vIL-10 retrovirus results in enhanced graft survival (27.6 days) whereas retroviral cellular IL-10 has no effect on prolonging survival (12.0 days) compared with controls (12.1 days).² These findings contrast the immunologic effects of vIL-10 and cellular IL-10 and suggest that local vIL-10 production, but not cellular IL-10, can suppress immune reactivity in response to alloantigen.

While vIL-10 gene transfer inhibited alloantigen-specific immune responses and prolonged graft survival, it was also associated with an unusual microscopic pathology. Viral IL-10-transduced allografts were characterized by a moderate mononuclear infiltrate, marked edema, and diffuse collagen deposition. These manifestations did not occur in syngeneic grafts perfused with the vIL-10 DNA-lipid complexes or allogeneic grafts perfused with vIL-10 antisense DNA-lipid complexes. This implies that the observed fibrosis was not a direct effect of the vIL-10 transgene product or lipid carrier, but that a response to an alloantigenic component is necessary. It might be postulated that vIL-10 inhibits only part of the allogeneic response and thus 'unmasks' a response resulting in the observed pathology. Little is known about the role of IL-10 in fibrosis. Reitamo *et al*²⁷ reported that cellular IL-10 can up-regulate elastin gene transcription, yet in the present study, we found no indication of abnormal elastin production. Other reports have indicated that cellular IL-10 inhibits postoperative intraperitoneal adhesion formation and activation of coagulation and fibrinolysis during human endotoxemia, suggesting that IL-10 down-regulates fibrotic processes.^{41,42} At

present, it is not clear what is inducing the fibrosis in our model, but one possible candidate may be TGF β . TGF β plays a role in promoting Th2 helper cells by inhibiting Th1 development, and is a prominent inducer of fibrosis.^{43,44} Because TGF β itself possesses many immunosuppressive properties, we have transduced cardiac allografts with viral and nonviral vectors encoding TGF β in the nonvascularized ear-heart model.^{1,23,24} In this system, we have demonstrated that TGF β gene transfer prolongs allograft survival (26.3 days versus 12.6 days for controls) but does not induce any apparent fibrosis.²³ Yet preliminary results with TGF β lipid-mediated gene transfer in the vascularized heart model demonstrated marked fibrosis of TGF β -transduced grafts (LD, JB, KB, unpublished). In addition, it was reported that *in vivo* gene transfer of TGF β into kidneys induced glomerulosclerosis, characterized by extracellular matrix accumulation.⁴⁵ Thus, future studies are aimed at determining the nature of the observed fibrosis. This is an important issue in that vIL-10-transduced grafts appear to succumb to edema and fibrosis rather than traditional immunologic rejection. Perhaps, if the fibrosis could be inhibited then vIL-10 gene transfer may be much more effective at prolonging graft survival.

Materials and methods

Animals

Female C57BL/6 (H-2^b) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) and BALB/c (H-2^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice used were between 8 and 12 weeks of age.

Medium

The culture medium used in these studies was DMEM supplemented with 1.6 mM L-glutamine, 0.27 mM L-asparagine, 10 mM Hepes buffer, 1.0 mM sodium pyruvate, 100 U/ml penicillin/streptomycin (all obtained from Gibco, Grand Island, NY, USA) and 5×10^{-5} M 2-ME (Sigma Chemical, St Louis, MO, USA). For limiting dilution microcultures, medium was supplemented with 10% heat inactivated fetal calf serum (Hyclone, Logan, UT, USA).

Plasmids and lipid

The plasmids pMP6AvIL-10(s) and pMP6AvIL-10(as) were constructed which encode the immunosuppressive cytokine viral IL-10, under the control of the HCMV promoter, in the sense and antisense orientation, respectively.⁴⁶ Plasmid DNA was combined with the cationic lipid N-(3-aminopropyl)-N,N-dimethyl-2,3-bis(dodecyl)-1-propaniminium bromide/dioleoyl phosphatidylethanolamine (γ AP DLRIE/DOPE), which was generously provided by VICAL (San Diego, CA, USA). DNA-liposome complexes were diluted in lactated Ringer's solution and incubated for 10 min at room temperature before being perfused into donor hearts.

Lipid-mediated gene transfer of cardiac allografts

Heterotopic cardiac transplantation was performed by anastomosing BALB/c donor hearts to the great vessels in the abdomen of C57BL/6 recipients as described by Corry *et al.*⁴⁷ For vIL-10 gene transfer, donor hearts were

perfused *in situ* by ligating the venae cavae to retain the perfusate. The distal aortic arch was then ligated and held in position by the suture to facilitate perfusion of the coronary vasculature via the aortic root with a 1 cc syringe and a 30-gauge needle. The donor heart was first flushed with 400 μ l of room temperature heparin (250 U/ml), then perfused with 250 μ l of room temperature DNA-liposome complexes in lactated Ringer's solution. Following perfusion, donor grafts were immediately harvested and placed in iced Ringer's solution for approximately 1 h and then transplanted without further perfusion or wash out. In this model, the transplanted heart resumes contractions until acutely rejected, which occurs in this strain combination in 8–9 days. Transplant function was monitored by daily abdominal palpation, and statistical comparison was performed with a Student's *t* test. For histological analysis, cardiac allografts were fixed in formalin, sectioned at 5 mm, and stained with either hematoxylin and eosin or trichrome for examination by light microscopy.

Anti-vIL-10 mAb treatment

Animals receiving vIL-10-transduced cardiac allografts were injected i.p. on days 0, 1 and 2 after transplant with 800 μ g of the purified rat anti-human IL-10 mAb JES3-19F1.1.1 (American Type Culture Collection, Rockville, MD, USA) that neutralizes vIL-10 but does not cross-react with murine cellular IL-10.⁴⁸

Recovery of heart infiltrating cells

Hearts were removed, minced and digested with 1 mg/ml collagenase A (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) for 30 min at 37°C. Heart infiltrating cells were washed free of collagenase and viable leukocytes enumerated by trypan blue exclusion.

Limiting dilution analysis of helper T lymphocytes

Two related techniques were used which distinguish *in vivo* stimulated or 'conditioned' alloantigen-reactive helper T lymphocytes (cHTL) from total precursor helper T lymphocytes (tHTL).⁴⁹ For tHTL, dilutions of recipient cells were added as responder cells to microtiter wells along with gamma irradiated (5000 R) allogeneic splenocyte stimulator cells and incubated for 16 h. CTLL-20 cells (an IL-2-dependent line) were then added directly to the microcultures, in order to detect IL-2 produced by the responder cells. The microcultures were incubated for an additional 24 h, including a 16 h terminal pulse with ³H-thymidine before harvesting by aspiration on to filter mats. For cHTL, recipient responder cells were gamma irradiated (2000 R) before addition to microcultures. After the addition of irradiated (5000 R) allogeneic stimulator cells, CTLL-20 cells were added at the initiation of culture and microcultures were pulsed with ³H-thymidine, incubated overnight and harvested. For both tHTL and cHTL, ³H-thymidine incorporation was determined on a scintillation counter. Individual microcultures were considered positive for IL-2 production if ³H-thymidine incorporation exceeded the mean plus 3 s.d. of ³H-thymidine incorporation in microcultures lacking responder cells.

Limiting dilution analysis of cytolytic T lymphocytes

LDA techniques were used which distinguish *in vivo* stimulated alloantigen-reactive cells (cCTL) from

unstimulated total precursor cells (tCTL) with the same alloantigen specificity.⁵⁰ For tCTL, dilutions of recipient cells were added to microtiter wells along with gamma irradiated (5000 R) allogeneic splenic stimulator cells and incubated for 7 days. To detect cytolytic activity, ⁵¹Cr-labeled BALB/c splenocyte concanavalin A blasts were added as target cells to the microcultures. Following a 4 h incubation, microculture supernatants were assayed for release of ⁵¹Cr in a gamma counter. For cCTL, the same 7 day culture conditions were employed except that irradiated (5000 R) syngeneic splenocytes were used in place of allogeneic splenic stimulator cells. Since no stimulating alloantigens were present in the modified LDA microcultures, only those CTL which had received an allogeneic stimulus before the analysis could demonstrate detectable cytolytic activity. Both tCTL and cCTL microcultures were considered cytolytic if observed chromium release was greater (mean plus 3 s.d.) than the chromium release observed in wells that contained target cells and stimulator cells but no responder cells.

Limiting dilution analysis

Minimal estimates of HTL or CTL frequency were obtained according to the Poisson distribution equation as the slope of a line relating the number of responder cells per microwell (plotted on a linear x-axis) and the percentage of microwells that failed to produce IL-2 or develop cytolytic activity, respectively (plotted on a logarithmic y-axis). The slope of this regression line was determined by computer using χ^2 minimization analysis, as described by Taswell.⁵¹ This analysis yields the minimal frequency estimate, the 95% confidence interval of the frequency estimate, and a χ^2 estimate of probability.

RNA isolation and cDNA synthesis

Cardiac allografts were homogenized in 1 ml RNAzol B (Teltest, Friendswood, TX, USA), which utilizes guanidinium thiocyanate to isolate total RNA. RNA was extracted with chloroform/isoamyl alcohol, precipitated with isopropanol, and washed with 70% ethanol. RNA was incubated with DNase at 37°C for 15 min then extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and washed with 70% ethanol. RNA was quantified using a spectrophotometer, and cDNA was obtained by random primer reverse transcription of 1 μ g of DNase-treated RNA.

DNA isolation

DNA was isolated by homogenizing tissues in 1 ml of TRIzol (GIBCO BRL-Life Technologies, Grand Island, NY, USA). DNA was extracted from the interphase and ethanol precipitated. DNA pellets were washed with 0.1 M sodium citrate in 10% ethanol, followed by 75% ethanol. DNA pellets were then resuspended in 8 mM NaOH and quantified using a spectrophotometer.

Polymerase chain reaction (PCR)

One microgram quantities of DNA or cDNA were amplified by PCR using two primers specific for the vIL-10 gene, 5'-ATGGAGCGAAGGTTAGTGGTC-3' (upstream) and 5'-ACTCTTGTTCTCACGCGAG-3' (downstream), which yield a 387 bp fragment. The PCR cycle used was 96°C for 15 s, 60°C for 30 s, and 72°C for 2 min. After 30 cycles, PCR products were run on 1.8% agarose gels and visualized by ethidium bromide staining.

Alloantibody isotype

P815 cells (H-2^d) were stained for FACS analysis using a 1:50 dilution of recipient serum as the primary antibody, followed by FITC-conjugated isotype-specific secondary antibodies. FITC-conjugated, affinity purified sheep anti-mouse IgM, IgG1 and IgG2a were obtained from The Binding Site (San Diego, CA, USA). Data are presented as the mean channel fluorescence determined on a Becton Dickinson FACScan (San Jose, CA, USA).

Acknowledgements

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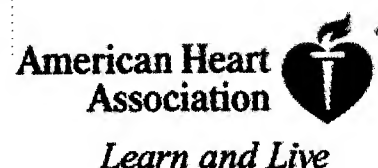
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Articles

In Vivo Gene Transfection of Human Endothelial Cell Nitric Oxide Synthase in Cardiomyocytes Causes Apoptosis-Like Cell Death

Identification Using Sendai Virus-Coated Liposomes

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Abstract

Background Nitric oxide (NO) has various actions on the cardiovascular system, although its pathophysiological significance in myocardial cells remains obscure. The aim of the present study was to identify direct NO actions on cardiomyocytes by gene transfection in vivo using a newly developed vector under physiological conditions.

Methods and Results Liposomes containing the β -galactosidase (β -gal) gene alone or with the human endothelial cell nitric oxide synthase (ecNOS) gene were coated with UV-inactivated Sendai virus and injected into the left ventricular wall of rat heart in vivo. Histological examination confirmed that the transfection efficiency was comparable to adenovirus-mediated

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transfection and that the new vector per se caused no inflammation. β -Gal expression was confined to cardiomyocytes between two intercalated discs, suggesting that the transfected gene did not permeate the discs. An immunohistochemical study showed that cotransfection of the ecNOS gene induced massive myocardial cell shrinkage in both transfected cells and the adjacent myocytes in a time- and dose-dependent manner. Histochemical findings in shrunk cells coincided with apoptosis as identified by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling. Electron microscopy of the lesion revealed myofibrillar degradation and accumulation of mitochondria but no apoptotic bodies. Pretreatment with the NOS inhibitor *N* ω -nitro-L-arginine methyl ester abolished these morphological alterations.

Conclusions The efficient expression of the human ecNOS gene in vivo suggests that NO or its toxic metabolite caused myocardial degradation, a part of which was compatible with apoptosis of the transfected cardiomyocytes themselves and the adjacent cells as a paracrine effect. These morphological features mimicked acute myocarditis or ischemic injury.

Key Words: genes • endothelium-derived factors • immunohistochemistry • myocardium • molecular biology

Introduction

Nitric oxide has a variety of actions on the cardiovascular system: vessel relaxation, inhibition of the proliferation of smooth muscle cells and endothelial cells, and suppression of platelet adhesion.^{1,2} The recent report of Balligand et al³ that ecNOS protein is constitutively expressed not only in coronary endothelial cells but in myocardial cells suggests autocrine and paracrine effects on cardiomyocytes, as is the case in vascular cells.^{4,5} Compared with NO released from activated macrophages, which might contribute to the pathogenesis of viral or bacterial myocarditis or septic shock,^{1,2} the pathophysiological significance of NO produced from myocardial cells is still obscure.

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In general, overexpression or knockout of a specific gene in myocardial tissue is of great use for the accurate elucidation of the actual function of its corresponding product. In a physiological setting, transmural pressure, passive stretching during diastole, and local coronary flow in the presence of several cytokines, which might modify gene expression, are preserved. Accordingly, local modulation of gene expression would be very significant for evaluation of the gene product.

In this study, the human ecNOS gene was successfully transfected by using Sendai virus-coated liposomes. This vector caused less inflammation than the adenovirus-mediated methods previously reported.^{6,7} Massive necrosis of myocardial cells, a part of which satisfied the criteria of apoptosis, is also described.

Methods

Plasmid Construction and Design of Vectors

The β -gal gene with a CMV promoter at an *Xba* I site and SV40 poly A signal at a *Bam*HI site was inserted into a pBluescript II KS+ plasmid (Stratagene). A 4.1-kb *Xba* I fragment of human ecNOS cDNA⁸ was inserted into a plasmid containing the CMV promoter with SV40 poly A signal (pcDNA 3, InVitrogen) at an *Eco*RI site.

Sendai virus (hemagglutinating virus of Japan)-coated liposomes were prepared.^{9,10} Nuclear protein (high-mobility group 1, 65 μ g) and histochemical marker (β -gal) gene alone (200 μ g) or with the ecNOS gene (200 μ g) were mixed and incubated for 1 hour at room temperature. The liposomes were then vortexed and sonicated with a lipid mixture (phosphatidylcholine, cholesterol, and phosphatidylserine) and incubated with UV-inactivated Sendai virus for 1 hour at 37°C.

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Gene Transfer In Vivo

After anesthetizing 300-g male Wistar rats (n=28) with pentobarbital sodium (25 mg/kg IP), the Sendai virus-coated liposomes were percutaneously injected into the left ventricular wall under the guidance of two-dimensional echocardiography. To examine the dose dependency of ecNOS gene transfection on histology, a high (50 μ g) or low (5 μ g) dose of ecNOS gene was administered.

NOS Inhibition by L-NAME and Its Verification

L-NAME was administered to rats in drinking water (12 mg/kg body wt⁻¹·d⁻¹, 100 mg/L) for 7 days before the transfection¹¹ to clarify the effects of L-NAME on NOS inhibition. After transfection, L-NAME treatment was continued in both the control (β -gal transfection alone) and

NOS gene (NOS plus β -gal transfection) groups for an additional 7 days. NOS inhibition was physiologically verified by measuring systemic pressure in the tail artery by sphygmomanometry and histologically examined by inspecting the medial thickening of coronary arteries.¹²

Light and Electron Microscopy

Rats were killed on days 3, 7, 10, and 14 ($n=4$ in each group) after the in vivo transfection. The hearts were excised, fixed in 2% paraformaldehyde in PBS for 4 hours at 4°C and sliced (2-mm thickness). After X-gal staining,¹³ the extent of β -gal expression was measured by obtaining the ratio of the X-gal-stained area to the cross-sectional area of the left ventricular wall. Paraffin-embedded 4- μ m-thick sections were then prepared for light microscopic assessment.

Other tissues fixed in PBS containing 2.5% glutaraldehyde and 2% paraformaldehyde for 12 hours at 4°C were postfixed in 2% osmium tetroxide for 2 hours at 4°C, embedded in epoxy resin 812, sectioned to 60-nm thickness, and stained with uranyl acetate for 15 minutes and lead citrate for 20 seconds at room temperature. Electron microscopy was performed with a Hitachi H 7000 electron microscope.

Immunohistochemical Assessment of Gene Expression and Macrophage Infiltration

Cryostat sections (6 μ m thick) of the hearts removed on day 7 after the transfection were fixed with 2% paraformaldehyde in PBS for 10 minutes. Endogenous peroxidase activity was quenched with 2% hydrogen peroxide in 60% methyl alcohol for 30 minutes at room temperature. The specimen was then permeabilized with 0.1% Triton X-100 in PBS for 20 minutes and incubated with either monoclonal antibody specific to ecNOS protein (5 μ g/mL, Transduction Laboratories) or monoclonal antibody specific to rat macrophage (2 μ g/mL, Serotec) overnight at 4°C. These sections were incubated with biotinylated rabbit anti-mouse IgG for 30 minutes at room temperature, and immunoproducts were visualized by using the avidin-biotin complex method (ABC kit, Vectastain). After rinsing, the slides were counterstained with Mayer's hematoxylin solution and mounted for light microscopy.

Assessment of Apoptosis

DNA was extracted from the transfected heart, and electrophoresis was performed in agarose gel to detect the ladder.¹⁴ For the in situ detection of apoptosis, cryostat sections fixed in 10% paraformaldehyde solution for 10 minutes at room temperature were treated according to the instructions for the apoptosis detection kit (ApopTag Plus, Oncor), which is a modification of the TUNEL method.¹⁵

Briefly, after endogenous peroxidase was quenched with 2% hydrogen peroxide in PBS for 5 minutes at room temperature, specimens were incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber for 1 hour at 37°C and then anti-digoxigenin peroxidase for 30 minutes at room temperature. They were then stained with diaminobenzidine substrate for 3 minutes at room temperature and counterstained in Mayer's hematoxylin solution for 1 minute at room temperature.

Statistical Analysis

For morphometry, pictures of five or six vision fields were taken at x400 magnification; observation fields were restricted to the area where the β -gal expression was evident in X-gal staining. Values are expressed as mean \pm SE. The results were considered significant if $P<.05$. Statistical significance was estimated among the various groups by using one-way ANOVA. Group-to-group comparisons were conducted by using Student's t test.

Results

Efficiency of Gene Transfection by Sendai Virus-Coated Liposomes

The expression of β -gal was clearly detected in the cross section of the transfected hearts (Fig 1 \blacksquare). Enzyme activity was visualized as blue color in the left ventricular wall in all hearts transfected with the β -gal gene alone or cotransfected with ecNOS genes. β -Gal expression was detected 3 through 14 days after transfection (Fig 2 \blacksquare). It peaked on day 7, when the extent of β -gal expression reached $11.6\pm 1.1\%$ ($n=4$) in cross section (Fig 2 \blacksquare).

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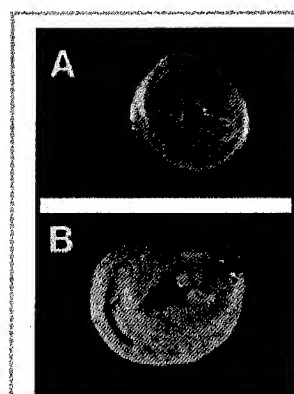


Figure 1. Photographs show expressions of β -gal gene alone (A) and β -gal plus ecNOS genes (B) on day 7 after in vivo transfection in the left ventricular wall of a rat. Blue indicates localized expression of β -gal activity.

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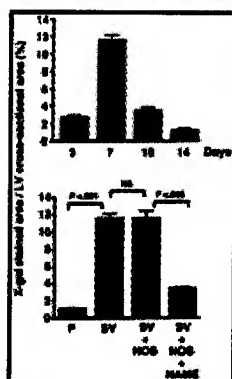


Figure 2. Bar graphs show time course of expression of β -gal activity after gene transfection in vivo to rat ventricular wall using a Sendai virus-coated liposome vector (top) and comparison of transfection efficiency for the expression of β -gal gene on day 7 (bottom). Ordinate shows the ratio of the area stained by X-gal to the cross-sectional area of the left ventricular (LV) wall. Rat heart was injected with plasmid gene alone encoding β -gal (P), Sendai virus-coated liposome vector containing β -gal gene plasmid (SV), or the same virus-coated liposome vector containing β -gal plus ecNOS gene plasmids (SV+NOS). The transfection efficiency of the ecNOS gene in Sendai virus-coated liposomes was also examined by pretreatment with the NOS inhibitor L-NAME (SV+NOS+NAME). Bars indicate mean \pm SE (n=4 in each group).

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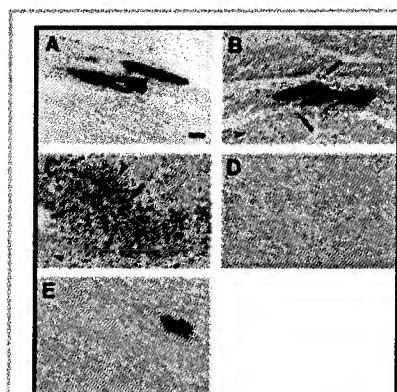
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In contrast, expression after the administration of plasmid DNA alone without Sendai virus-coated liposomes attained only $1.1\pm0.1\%$ (n=4, $P=.0003$ versus virus-coated liposome vector; Fig 2B). Cotransfection of the ecNOS gene did not affect the extent of β -gal expression ($11.6\pm1.7\%$, n=4; Figs 1B and 2B). However, when animals were treated with L-NAME to examine the effect of NOS inhibition, β -gal expression decreased even when Sendai virus-coated liposome vectors were used in the cotransfection with ecNOS ($3.4\pm0.4\%$, n=4, $P=.0022$ versus without treatment; Fig 2B) or β -gal transfection alone ($3.6\pm0.2\%$, n=4). It is unclear why β -gal expression decreased with L-NAME treatment. Changes in systemic or coronary pressure and circulation with L-NAME treatment may influence β -gal gene expression.

Histological and Physiological Findings of β -Gal Expression and ecNOS Transfection With or Without L-NAME Treatment

Microscopic examination revealed characteristic features of gene expression after transfection (Fig 3B). β -Gal activity was detected throughout the entire myoplasm of the transfected cells and was clearly distinguished by an intercalated disc from adjacent cells where β -gal was not expressed. Infiltration of white blood cells in the transfected portion was slight, and the inflammatory process due to vector administration was much less than for the adenovirus-mediated method (Fig 3A).^{6,7}



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Figure 3. Histological photomicrographs of rat hearts transfected in vivo with β -gal gene alone (A), β -gal plus ecNOS genes on day 7 after transfection (B and C), ecNOS gene alone (D), or L-NAME pretreatment before cotransfection with the two genes (E). The ecNOS gene was transfected at low (5 μ g; B) and high (50 μ g; C through E) doses. Nucleus and myoplasm were counterstained by using Kernechtrot's method. Note two types of elongated, small cells in transfected myocardium; blue (arrows) cells are detected at the center of the degraded region, and nonstained (arrowheads) cells are visible at the periphery of the gene-transfected region (bar=20 μ m; magnification x400).

Furthermore, ecNOS gene transfection together with the β -gal gene demonstrated markedly different findings from the marker gene transfection alone (Fig 3B and 3C). At the low dose (5 μ g) of the ecNOS gene no or minimal lesions were documented (Fig 3B), but at the high dose (50 μ g) a clear border was delineated between the intact cardiac tissue and the injured area (Fig 3C). The lesion corresponded to the transfected area of the ecNOS gene because β -gal staining was restricted to that lesion (Fig 3B and 3C). The sizes of most cells in the transfected area were greatly reduced, and the myoplasm of the degenerated cells was much decreased (Fig 3C).

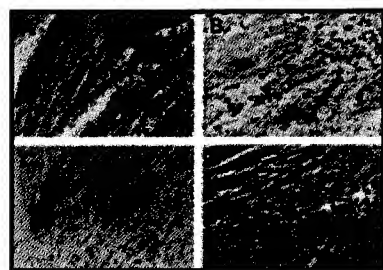
Small cells in the lesion could be classified into four types according to the shape (elongated or round) and stain (blue or not stained). Elongated cells stained blue were scattered throughout the necrotic lesion (arrows in Fig 3C), while elongated, unstained cells were detected in the periphery (arrowheads in Fig 3B and 3C). These elongated cells may have originated from denatured myocardial cells, while the round cells might be infiltrating macrophages, as identified later. For ecNOS gene transfection alone without the marker (β -gal) gene, the injured area had the same changes as observed for cotransfection (Fig 3D).

The pathological alterations in the NOS gene group were markedly reduced with L-NAME treatment (Fig 3E) because the number of shrunk cells decreased to the same level as the control. The cardiomyocytes transfected with β -gal preserved their original histological structure. These findings suggest that the pathological degeneration after ecNOS gene transfection was due to NO or its metabolites. The medial thickening of coronary arteries was not histologically obvious in the L-NAME-treated group, probably because the dose and period were smaller than in the previous report.¹² Accordingly, these drastic alterations in the transfected lesion would be due to the ecNOS gene and not the β -gal gene.

L-NAME treatment confirmed the NOS inhibitory effect since the systolic pressures of rats increased from 117 ± 3 to 158 ± 3 mm Hg ($n=4$, $P<.01$) in the control group (β -gal transfection alone) and from 120 ± 2 to 160 ± 2 mm Hg ($n=4$, $P<.01$) in the NOS gene group (NOS plus β -gal transfection).

Characterization of the Transfected Lesion

Immunohistochemical staining with antibody specific to ecNOS demonstrated endogenous NOS protein on myocardial cells³ as well as endothelial cells in coronary arteries (Fig 4A). In overtransfected myocardial cells, additional staining was visualized as thick staining. Cotransfection of the ecNOS and β -gal genes resulted in the mixed color of brown (immune complex with ecNOS protein) and blue (β -gal activity; arrows in Fig 4A). Small elongated cells without blue staining (arrowheads in Fig 4A) were also documented at the border between the degraded lesion and normal myocardial cells. Occasionally, either β -gal activity or ecNOS protein was clearly detected on normal-sized myocardial cells, and sarcomeres were clearly visible in these cells. This might be due to the selective transfection of one of the two genes in each myocardial cell.



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Figure 4. Immunohistochemical staining for ecNOS protein (A) and rat macrophage (B), in situ apoptotic reaction (C), and azan staining (D). A through D, Blue staining indicates expression of β -gal activity. A, Arrows indicate small cells stained blue; arrowheads, atrophic cells not stained blue; and stars, overexpression of ecNOS protein after in vivo gene transfection in addition to constitutive ecNOS protein in cardiomyocytes.² C, Brown nucleus (arrows) indicates reaction to TUNEL detection. Note that positively stained cells do not always accompany apoptotic bodies (bar=20 μ m; magnification \times 400).

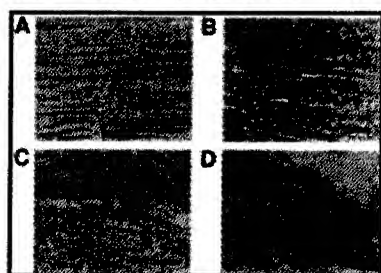
The infiltration of a large number of macrophages was detected with specific antibody in the injured area, indicating that a portion of the round small cells found in the transfected lesion (Fig 4B²) represent invading leukocytes and macrophages.

Recent studies have indicated that apoptosis occurs with cell necrosis.¹⁶ End labeling of fragmented DNA with the TUNEL method demonstrated that 0.5% of the nuclei in small cells in the degenerated lesion were positive for the apoptotic reaction in situ, whereas none of the control myocardial cells were positive (Fig 4C²). All cells that were positively stained shrank in volume.

Azan staining revealed deposition of collagen fibers in the necrotic lesion (Fig 4D²), suggesting that the degenerative process after the ecNOS gene transfection was accompanied by fibrosis. The pathological findings of leukocyte invasion and fibrosis were similar to those of the inflammatory lesion in myocardial infarction at the subacute stage or myocarditis of viral or bacterial origin.

Electron Microscopic Assessment

Electron microscopy of the untransfected area showed no pathological deterioration, and myofibrillar array and mitochondrial structure were both well preserved (Fig 5A²). However, in the transfected area, mitochondrial accumulation and swelling were found between thin myofibrils (Fig 5B²). The mean numbers of these mitochondria in the transfected and untransfected areas, respectively, were 95 ± 7 and 82 ± 5 per 100 μ m² ($P < .05$). The mean diameter of these mitochondria in the transfected area was 1.16 ± 0.04 μ m, compared with 0.91 ± 0.04 μ m ($P < .01$) in the untransfected area. The mitochondria were round or elliptical in the untransfected area; some of the mitochondria between the sparse myofibrils in the transfected area were deformed and shaped like confetti.



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Figure 5. Electron photomicrographs of a control region distant to the transfected site in the left ventricular wall (A) and the region transfected with human ecNOS gene at the high dose (50 μ g) together with the β -gal gene (B through D) on day 7. Note that typical features of apoptosis are lacking, including apoptotic bodies and chromatin condensation (C) and a clear contrast of the lesion bordered by an intercalated disc (D) (bar=2 μ m; magnification \times 3000).

In addition to these mitochondrial abnormalities, various changes within the myocytes were observed, including intracellular edema, depletion of glycogen granules, and sparse myofilament arrays (Fig 5C²). However, the integrity of the cytoplasmic membrane was preserved, with both apoptotic bodies and chromatin condensation being absent (Fig 5C²). As described in the light microscopic study (Fig 3A²), a clear contrast bordered by an intercalated disc was observed in two myocytes (Fig 5D²). In other words, most of the mitochondria were destroyed on one side of the intercalated disc.

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We have made five major observations. First, in contrast to adenovirus vector,^{6,7} the newly developed Sendai virus-coated liposomes were very useful for the efficient transfection of gene in vivo with little associated inflammation. Second, both the reporter gene β -gal and the physiologically significant gene ecNOS were transfected to cardiomyocytes in living animals. Third, expression of the β -gal gene was limited to the cardiomyocytes between two intercalated discs, providing a clear contrast with adjacent cells. Fourth, overexpression of the ecNOS gene caused unique cell degeneration, including a reduction in myoplasm volume, mitochondrial accumulation, collagen deposition, and macrophage infiltration. Finally, these morphological alterations were ameliorated with L-NAME pretreatment.

Biological Characteristics of Sendai Virus-Coated Liposome Vector

To elucidate the specific function of an expressed gene, gene transfer is superior to transgenic animals for the following reasons. When the gene product is systemically expressed, it may obscure the local function. This is especially true when the gene product is essential for maintaining cell viability or the overexpressed product is lethal to the animal. Local expression or knockout of a specific gene is of great significance for identifying the physiological function of the gene product in situ. In this setting, the vector should not cause a secondary effect.

Adenovirus vector is not appropriate for this purpose because the vector itself causes local inflammation.^{6,7} Sendai virus-coated liposomes were vastly superior to adenovirus-mediated transfection because Sendai virus has no pathogenicity in either humans or rodents. UV-irradiation of Sendai virus is beneficial for preventing infection in other species, and the process does not weaken the transfection efficiency of the liposomes. Local administration of the newly developed vector in vivo induced no harmful action other than the needle puncture (Fig 3A \oplus). Furthermore, expression of the β -gal gene was confined to myocytes between two intercalated discs, suggesting that the transfected gene did not permeate the discs. Comparison of transfected cardiomyocytes and nontransfected adjacent cells with electron microscopy (Fig 5D \oplus) was particularly useful.

Morphological Alterations After ecNOS Gene Transfection and Relation to Apoptosis

The classic criteria for apoptosis^{17,18,19} include a reduction of cytoplasm volume, which was shown in most cells in the degraded lesion (Fig 3B \oplus and 3C \oplus), detection of apoptotic reaction as identified by end labeling of fragmented DNA (Fig 4C \oplus), and no disruption of the cytoplasmic or nuclear membranes of the degraded cells as assessed by electron microscopy (Fig 5C \oplus). Other characteristics of apoptosis not observed in the present study include the presence of apoptotic bodies, chromatin condensation, and a DNA ladder after gel electrophoresis. Thus, the present data satisfied some but not all of the features of typical apoptosis.

Pathological examination also demonstrated an inflammatory process in the transfected myocardium. Double staining of β -gal activity and ecNOS protein (Fig 4A \oplus) or macrophages (Fig 4B \oplus) using specific antibodies, TUNEL analysis of apoptosis (Fig 4C \oplus), and azan staining (Fig 4D \oplus) revealed coexisting myocardial cell necrosis, apoptosis, and lymphoplasmacytic infiltration in the transfected myocardium. Pinsky et al²⁰ have indicated that NO production in vitro from activated macrophages causes cytotoxic action on myocardial cells in tissue culture. Their results, together with the present findings, suggest that an inflammatory process does not exclude the possibility of apoptosis.

Pathological Significance of Injured Cells

Occasional myocytes located at the periphery of the transfected lesion showed degradation without expression of ecNOS protein (Fig 4A \oplus). This finding may indicate that the cells transfected by the ecNOS gene and adjacent nontransfected cells were injured by NO or its secondary metabolites, such as peroxynitrite (ONOO⁻), in an autocrine or paracrine manner, respectively. This scenario is likely since NO is a diffusible gas and is actually excreted from NO-producing cells.^{5,20}

A very small number of atrophic myocytes stained positively for the apoptotic reaction in cryostat sections in situ, whereas none of the control myocardial cells were positive (Fig 4C \oplus). All cells that stained positively by the TUNEL method had decreased cell volumes. It should be emphasized that nucleus fragmentation, which is characteristic of apoptosis, was not detected at all. The heterogeneity of the apoptotic reaction among the atrophic cells suggests that a stage in the degradation process is required for apoptotic reaction; hence, the other denatured cells could not react to TUNEL staining.

The electron microscopy results revealed mitochondrial accumulation and swelling of the injured cells (Fig 5B \oplus) compared with control cells in the untransfected portion of the same myocardial tissue (Fig 5A \oplus). This indicates that mitochondria in a limited area were affected by ecNOS gene transfection and that the overexpression of ecNOS did not cause any morphological changes in myocytes distant from the transfected lesion. Impairment of oxidative phosphorylation and energy metabolism might lead to failure of the mitochondrial cation pump, and subsequently progressive swelling before mitochondrial rupture.²¹ The accumulation and swelling of mitochondria in degenerated lesions may be a defense mechanism against free radicals, including ONOO⁻, because mitochondria are one of the main sources of toxic free radicals.²²

Hibbs et al²³ report that activated macrophages cause inhibition of mitochondrial respiration.

In addition to the mitochondrial abnormalities, other changes within the myocytes were observed, including intracellular vacuole formation, depletion of glycogen granules, and sparse myofilament arrays. These findings suggest deterioration of the myofibrils and their subsequent absorption (Fig 5C-E). Most damaged cells with mitochondrial abnormalities did not exhibit typical apoptotic bodies or dense chromatin, which are characteristic of apoptosis.¹⁹ Although these changes satisfied some of the oncosis criteria proposed by Majno and Joris,²⁴ the present findings lacked blebbing and increased permeability of the cytoplasm membrane.

Conclusions

The findings of the present study suggest that overexpression of ecNOS by in vivo gene transfection probably produced NO or its toxic metabolites, which caused unique myocardial cell death. These cell injuries fulfilled some but not all of the criteria for apoptosis in the transfected cardiomyocytes themselves and in the adjacent cells as a paracrine effect. It would be attractive to assume that similar cell death may be involved in the progression of acute myocardial infarction, viral myocarditis, the development of cardiomyopathy, or the regression of cardiac hypertrophy.

Selected Abbreviations and Acronyms

CMV	= cytomegalovirus
ecNOS	= endothelial cell nitric oxide synthase
β -gal	= β -galactosidase
L-NAME	= N ω -nitro-L-arginine methyl ester
NO	= nitric oxide
NOS	= nitric oxide synthase
TUNEL	= terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling
X-gal	= 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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Footnotes

Drs Kawaguchi and Shin contributed equally to the present study.

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EXHIBIT I

Liposome-Mediated Gene Transfer into Normal and Dystrophin-Deficient Mouse Myoblasts

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Abstract: A range of tissue types has now been targeted for development of gene therapeutic procedures both to correct genetic defects and to treat acquired disease. In particular, skeletal muscle holds great importance, not exclusively for the treatment of inherited muscle disorders but also as a platform for the expression of heterologous recombinant proteins, destined to immunise the host or to serve some systemic therapeutic goal. With respect to the X-linked myopathy Duchenne muscular dystrophy (DMD), several gene therapy protocols are being developed that focus on complementing primary genetic defects in the DMD gene by introducing copies of recombinant gene constructs into muscle cells both *ex vivo* and *in vivo*. In the present study the potential use of a range of polycationic liposomes as physical gene delivery systems for skeletal muscle has been examined. Using a LacZ reporter gene under optimised conditions up to 40% transfection efficiencies were obtained with the mouse myoblast cell line C2C12. With primary cultures of normal and dystrophin-deficient *mdx* mouse muscle, up to 10% transfection efficiency was obtained with reporter gene constructs, and high levels of recombinant human dystrophin expression were observed following transfer of dystrophin cDNA gene constructs. These *in vitro* studies indicate that cationic liposomes can be used to deliver recombinant genes to muscle cells at high efficiency and form a basis to expand investigations into *in vivo* expression of recombinant dystrophin protein either by direct intramuscular gene transfer or via implantation of transfected myoblasts. **Key Words:** Dystrophin—Liposome—Gene transfer.

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The recessive X-linked myopathy Duchenne muscular dystrophy (DMD) is caused by null mutations in the gene encoding dystrophin, a 427-kDa cytoskeletal component of skeletal, cardiac, and smooth muscle cells (Ahn and Kunkel, 1993). The disease affects one in 3,500 boys, causing progressive degeneration of limb and axial musculature and death in the second or third decade as a consequence of cardiac and respiratory impairment (Emery, 1993). Recently, several genetic complementation strategies have been proposed as potential therapeutic treatments for DMD, including

the transplantation of healthy donor myoblasts (Partridge et al., 1989; Gussoni et al., 1992) and direct *in vivo* transfer of recombinant dystrophin genes by intramuscular injection of plasmid (Acsadi et al., 1991), retroviral (Dunckley et al., 1992, 1993), or adenoviral (Ragot et al., 1993; Vincent et al., 1993) vectors. Direct plasmid injection is the simplest and safest gene transfer approach but is of too low efficiency for clinical efficacy. The alternative viral-mediated systems, while allowing potentially high gene transfer efficiency, suffer from limited capacities for foreign DNA, requiring the use, for example, of severely truncated dystrophin cDNAs, and their potential clinical use raises important safety issues (Cornetta et al., 1991). Approaches to enhance the efficiency of nonviral DNA-mediated gene transfer systems have included electroporation (Neumann et al., 1982), particle bombardment (Yang et al., 1990), DNA precipitation (Loyter et al., 1982), and liposomes (Felgner et al., 1987; Felgner and Ringold, 1989) and have been widely used *in vitro*. More recently, cationic liposome vesicles have also been used successfully to transfer genes into a range of cell types *in vivo* (Debs et al., 1990; Alton et al., 1993; Nabel et al., 1993). This method of transfection is dependent on ionic interactions between newly formed lipid–DNA complexes and the target cell membranes, leading to endosomal uptake into the cytosol and subsequent appearance of active genetic material in the nucleus. The route and mechanism whereby exogenous DNA attains the nuclear compartment following internalisation remain poorly understood but may be in part cell cycle depen-

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Abbreviations used: DMD, Duchenne muscular dystrophy; DMEM, Dulbecco's modified Eagle's medium; DOPE, dioleoyl-phosphatidylethanolamine; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethyl sulphate; HHS, heat-inactivated horse serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate.

dent, although successful transfer into tissues with low mitotic activity such as lung epithelium has been described. In addition, the efficiency and cytotoxicity of a wide range of liposome formulations have been shown to be highly cell type specific. With the aim of developing enhanced dystrophin gene delivery systems for skeletal muscle, we have evaluated and optimised a range of liposome formulations for the transfer of recombinant reporter genes and full-length dystrophin cDNA constructs into cultured mouse muscle cells from the C2C12 cell line as well as from normal and dystrophin-deficient *mdx* mice.

MATERIALS AND METHODS

Cell culture

C2C12 cells are a subclone of the C2 cell line isolated from mouse skeletal muscle (Yaffe and Saxel, 1977). Primary myoblast cultures were obtained by sterile enzymatic dissociation (Walsh and Ritter, 1981) of hind limb muscle from 5–6-week-old *mdx* and normal C57/B10 mice. C2C12 myoblasts were seeded at varying densities onto 35-mm-diameter tissue culture dishes (Nunc), whereas primary myoblasts were seeded onto 35-mm-diameter laminin-coated dishes as described (Dunkley et al., 1992). Myoblast cultures were allowed to proliferate in growth medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) foetal calf serum and 2 mM glutamine. For myotube cultures, the medium overlying confluent myoblast cultures was replaced with DMEM containing 5% (vol/vol) heat-inactivated horse serum (HIHS) and 2 mM glutamine, to induce fusion.

Plasmid DNA constructs

Eukaryotic expression plasmids containing either the *Escherichia coli* β -galactosidase gene [pRSVLacZ (Acsadi et al., 1991)] or full-length human dystrophin cDNA [pRSVDy (Acsadi et al., 1991; Dickson et al., 1991)] driven by the Rous sarcoma virus promoter. Supercoiled plasmid was purified by double CsCl₂ density gradient centrifugation.

Cationic liposomes

Four different liposome formulations were examined for their use in plasmid DNA transfection: LipofectAMINE, a 3:1 (wt/wt) formulation of 2,3-dioleoyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propylammonium trifluoroacetate and dioleoylphosphatidylethanolamine (DOPE; GIBCO; 2 mg/ml); Lipofectin, a 1:1 (wt/wt) formulation of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride and DOPE (GIBCO; 1 mg/ml); LipofectACE, a 1:2.5 (wt/wt) formulation of dimethyl dioctadecylammonium bromide and DOPE (GIBCO; 1.4 mg/ml); and *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethyl sulphate (DOTAP; Boehringer Mannheim; 1 mg/ml). All liposome stocks were stored in aqueous solution at 4°C until use.

Transfection of C2C12 and primary muscle cultures

Myoblasts were seeded at varying densities ($0.5\text{--}2 \times 10^5$) onto 35-mm-diameter tissue culture dishes, incubated overnight, and transfected the following day, when the medium was replaced with 0.8 ml of serum-free DMEM supplemented with 2 mM glutamine for 30 min. Stock liposome

reagents (2.5–30 μ g) and plasmid DNAs containing the recombinant gene of interest (0.75–32 μ g) were diluted independently in 100 μ l of 150 mM NaCl and then combined and overlaid onto the monolayer of cells in the presence of the serum-free medium. After 1–10 h at 37°C the transfection mixture was replaced with standard DMEM containing HIHS. Cultures were assayed by β -galactosidase histochemistry, dystrophin immunocytochemistry, or western blot analysis at varying intervals posttransfection (see below).

β -Galactosidase histochemistry

Cultures were stained for transgene expression essentially as described by Sanes et al. (1986). In brief, samples were washed three times with phosphate-buffered saline (PBS) before fixation in the same buffer containing 1 mM MgCl₂ and 0.5% glutaraldehyde for 10 min at room temperature. Further permeabilisation and three 5-min washes were performed in PBS containing 1 mM MgCl₂ and 0.02% Nonidet P-40. Finally, samples were incubated overnight at 37°C in PBS containing 1 mM MgCl₂, 33 mM K₄Fe(CN)₆, 33 mM K₃Fe(CN)₆, and 0.1% 5-bromochloro-3-indolyl- β -D-galactopyranoside (X-gal).

Immunofluorescence staining for dystrophin

Cultures were fixed in methanol (–20°C, 5 min) and then incubated sequentially for 1 h in PBS containing 1% foetal calf serum with first the species-specific monoclonal antibody 12B2 to human dystrophin (Huard et al., 1993) followed by biotinylated sheep anti-mouse IgG and streptavidin–Texas Red (Amersham). Stained cells were viewed on a Zeiss microscope equipped with phase-contrast and epifluorescence optics.

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and western blotting

Myotube cultures (35-mm-diameter dishes) were rinsed twice with PBS and harvested in 100 μ l of SDS sample buffer [75 mM Tris (pH 6.8), 10% (wt/vol) SDS, 100 mM dithiothreitol, 0.1% bromophenol blue, 20% glycerol, 2 ng/ml of phenylmethylsulphonyl fluoride, 2 μ g/ml of EDTA, 5 mg/ml of antipain, 5 mg/ml of aprotinin, and 500 μ g/ml of leupeptin]. Samples were triturated through a 25-gauge needle, heated in a boiling bath for 3 min, and microfuged for 2 min. Comparative samples were subjected to SDS-PAGE using a 6% resolving gel, transferred to an Immobilon-P transfer membrane, and probed with a mouse monoclonal anti-rod domain dystrophin antibody (6D3) as previously described using an enhanced chemiluminescence detection system (ECL; Amersham) (Nicholson et al., 1989; Noursadeghi et al., 1993).

Evaluation of transfection efficiency

In experiments using the LacZ reporter gene constructs the transfection efficiency was quantified by counting the number of blue β -galactosidase-stained myoblasts or myotubes as a proportion of the total number using an eyepiece graticule. Four randomised fields were counted for each individual culture, and data points were evaluated as mean \pm SD values of counts performed on replicate independent cultures.

RESULTS

Optimisation of transfection

Initial studies were performed using four liposome formulations—LipofectAMINE, Lipofectin, Lipofect-

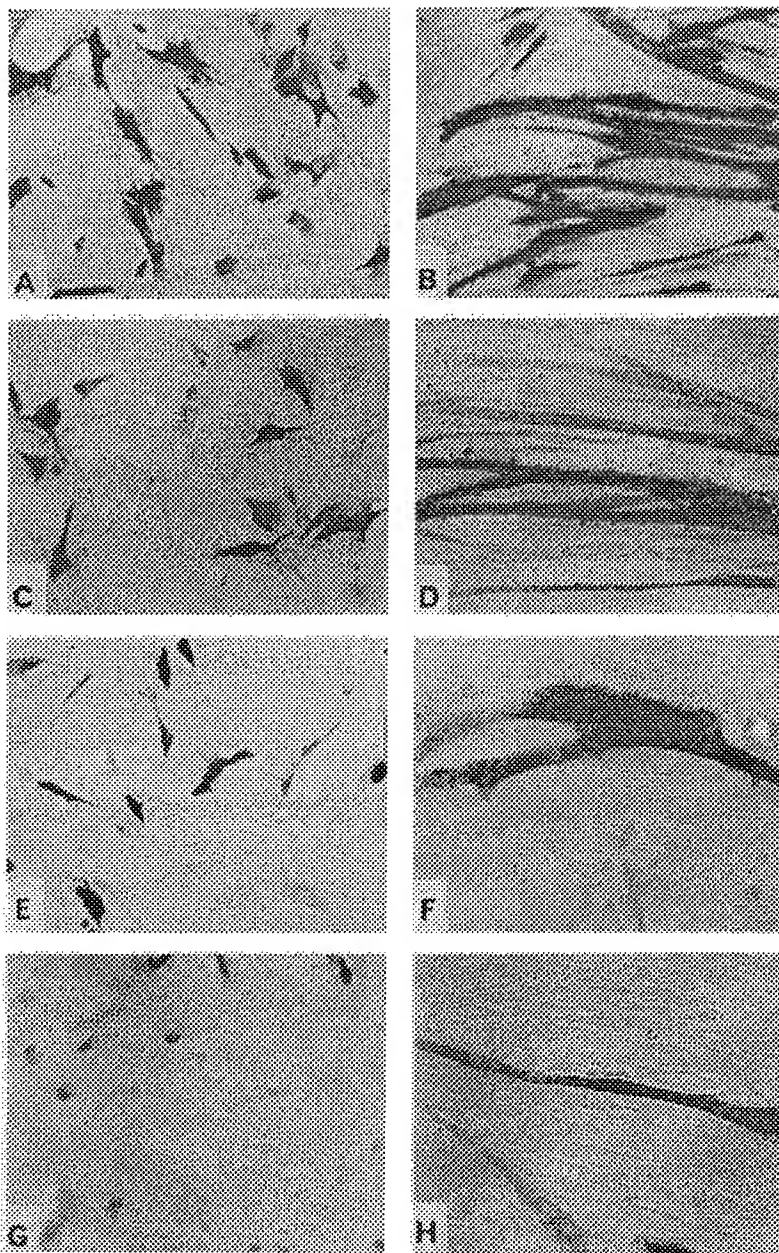


FIG. 1. Qualitative comparison of liposome reagents. C2C12 myoblast cultures were transfected using each individual reagent optimised lipid:DNA ratios, plating cell density, and incubation time parameters. **A, C, E, and G:** Expression pattern of the transfected recombinant β -galactosidase gene 48 h posttransfection. **B, D, F, and H:** Reporter gene expression 5 days posttransfection. Qualitative differences indicate that LipofectAMINE was the best of the four reagents tested (**A** and **B**), in a gene transfer using 10 μ g of reagent and 2 μ g of plasmid DNA (pRSVLacZ) transfected into C2C12 cultures plated at a density of 1.5×10^3 per 35-mm-diameter plate for 6 h. **C** and **D** show the results of the optimal transfection with the DOTAP reagent using 10 μ g of reagent and 8 μ g of DNA, with all other parameters being identical to the LipofectAMINE reagent transfection. **E** and **F** show the outcome of the LipofectACE transfection, and **G** and **H** show the expression in the Lipofectin-transfected culture. In both cases 10 μ g of reagent when complexed to 1 μ g of DNA and transfected for 6 h with a plating density of 1.5×10^3 was found to be optimal. $\times 225$.

ACE, and DOTAP—to transfect the pRSVLacZ into myogenic C2C12 cells. Transfection efficiencies were maximised by optimising the lipid:DNA composition ratios, incubation period, and cell density parameters of the transfection protocol (Fig. 1). Expression of β -galactosidase was detectable when cultures were stained up to 10 days posttransfection, and routine analyses were performed at 5 days posttransfection when myotube formation was largely complete. Fixing

the quantity of DNA in the transfection mixture, liposome concentrations were initially optimised, and with each formulation a peak transfer efficiency was obtained using 10 μ g of reagent (Fig. 2A). Transfecting cultures with levels of liposomes exceeding 10 μ g resulted generally in reduced efficiency along with increasing cellular toxicity. Optimal DNA:lipid ratios were then determined by varying the amount of plasmid DNA complexed with a fixed liposome quantity

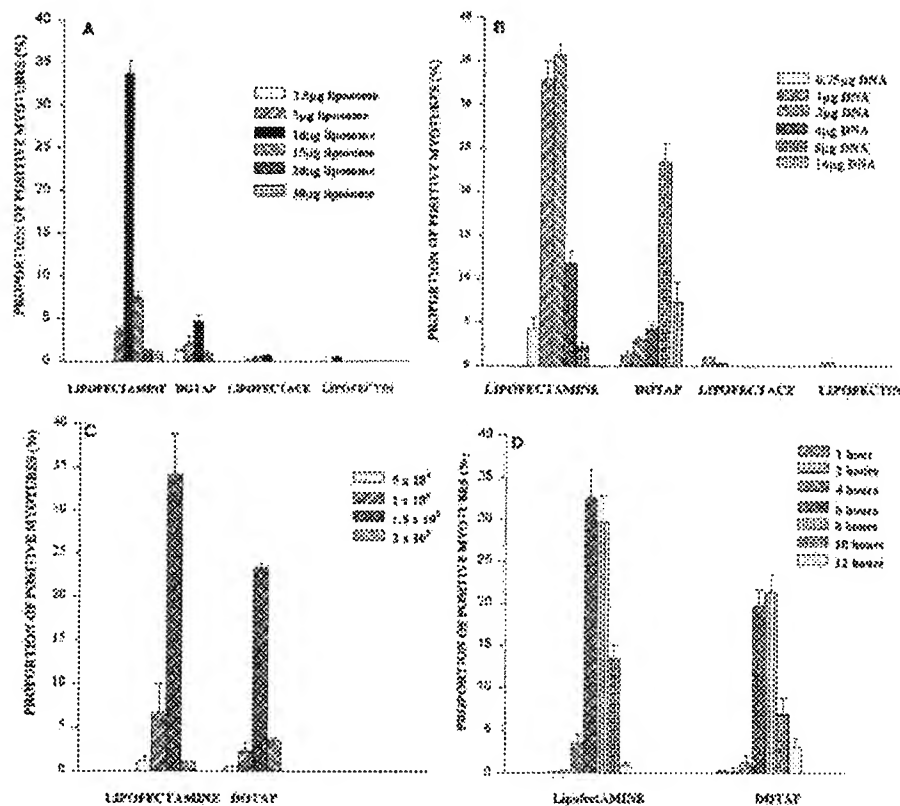


FIG. 2. Optimisation of the transfection protocol. Efficiency of gene transfer was maximised by using C2C12 cultures to optimise the four most important transfection parameters. **A:** Effect of varying liposome concentration. C2C12 myoblasts (1.5×10^5) were plated onto 35-mm-diameter dishes and left to grow overnight. The following day cultures were transfected for 6 h with 1 μ g of pRSVLacZ complexed to the varying amounts of liposome indicated. **B:** Effect of varying DNA concentration. Keeping the optimal liposome concentrations fixed for all reagents (10 μ g), DNA concentration was varied as shown while cell density and incubation time remained unchanged. Owing to poor expression with both the LipofectACE and the Lipofectin reagents only the DOTAP and LipofectAMINE reagents were further examined. **C:** Effect of plating cell density. Using established optimal lipid and DNA amounts (10 μ g of LipofectAMINE and DOTAP complexed to 2 and 8 μ g of DNA, respectively) while maintaining incubation time at 6 h, overnight-grown C2C12 cultures plated at the densities indicated were transfected the following day with pRSVLacZ. **D:** Effect of varying transfection time. Fixed liposome:DNA ratios and optimised plating cell density of 1.5×10^5 were maintained while incubation time was varied as shown. Assessment of transfection efficiency was made by counting the proportion of positive blue myoblasts. In subsequent transfections a plating cell density of 1.5×10^5 cells per 35-mm-diameter dish and 6 h of incubation were used when either 10 μ g of LipofectAMINE and 2 μ g of DNA or 10 μ g of DOTAP and 8 μ g of DNA were used to transfect C2C12 myoblast cultures.

of 10 μ g (Fig. 2B). Of the four liposome formulations, gene transfer was maximal using the LipofectAMINE reagent with efficiencies up to 40% (DNA:lipid ratio of 1:5). Using the DOTAP reagent, maximal efficiencies in the range 15–20% were obtained with DNA:lipid ratios of 1:1. Both the Lipofectin and LipofectACE reagents yielded only very low transfection efficiencies under all conditions examined. For this reason only the LipofectAMINE and DOTAP reagents were examined in subsequent experiments, where protocols were further optimised by individually varying the interval cells were exposed to the transfection mixture and cell density at which target cultures were initially seeded. Maintaining fixed DNA and liposome levels, peak efficiencies were observed after a 6-h incubation

with LipofectAMINE or DOTAP (Fig. 2C) with an optimal plating cell density of 1.5×10^5 (Fig. 2D). Similar results were obtained whether cultures were examined for β -galactosidase expression 5 days after transfection, when myotube formation was largely complete, or 2 days after transfection, when cultures consist predominantly of mononucleated myoblasts (Fig. 3).

Recombinant dystrophin gene transfer into C2C12 myoblasts

Using the optimised transfection parameters and replacing the LacZ gene construct with a human dystrophin cDNA construct (pRSVDy), we then examined expression of the recombinant dystrophin in cul-

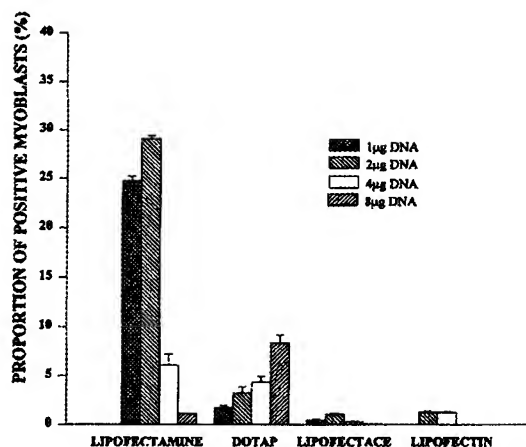


FIG. 3. Quantitative assessment of transfection efficiency based on reporter gene expression in transfected C2C12 myoblasts. For each 35-mm-diameter dish 1.5×10^5 cells were transfected for 6 h with 10 μ g of each liposome reagent complexed to the amounts of DNA indicated. Transfection mixture was replaced with growth medium after 6 h. Cultures were histochemically assayed for reporter gene expression 48 h later.

tured myotubes. The C2C12 murine cell line, although shown to have an impairment in dystrophin gene transcription (Klamut et al., 1990), is known nevertheless to express low levels of endogenous dystrophin (Nour-sadeghi et al., 1993). To distinguish endogenous murine dystrophin from that potentially being expressed from the recombinant gene, the protein was detected using a human species-specific antidystrophin antibody, 12B2. Both the DOTAP and LipofectAMINE reagents were used to transfect C2C12 myoblast cultures with the dystrophin plasmid. These cultures were grown for between 10 and 12 days and allowed to differentiate terminally into myotubes before being probed with the 12B2 antibody for recombinant dystrophin expression. Immunofluorescence staining patterns demonstrated expression of the dystrophin transgene with the recombinant protein localised to the plasma membrane of the myotubes. As shown in Fig. 4B and D, dystrophin-positive myotubes could be seen among a control background of unstained cells. In addition, transfected cultures were harvested for western blotting analysis 48 h posttransfection (Fig. 5). The resulting western blot transfers clearly identified high levels of the 427-kDa recombinant dystrophin protein in samples derived from cultures transfected with either the DOTAP (lanes 3–5) or LipofectAMINE (lanes 6–8) reagent under optimal conditions, with no detectable endogenous band in control cultures (lane 2).

Transfection of myoblasts in primary culture

One of the aims of the present study was to examine expression of recombinant dystrophin in muscle cells cultured from the dystrophin-deficient *mdx* mouse. Re-

examination of the transfection parameters was necessary to account for potential differences between myoblasts in primary dissociation cultures and the immortalised C2C12 cell line. Reverting back to the reporter gene construct pRSVLacZ, all four liposome reagents were again studied for their ability to transfect primary cultures of mouse myoblasts. Previous studies had suggested that both the Lipofectin and the LipofectACE reagents may be particularly suited for the primary culture environment (Jarnagin et al., 1992; Jiao et al., 1993). In general, the optimised protocols observed for primary myoblast transfection were similar to those determined for the C2C12 cell line with all four liposome formulations, with, however, marked differences in the absolute levels of gene transfer. Maximal transfection efficiencies of 5–10% were observed in the primary cultures transfected with the LipofectAMINE and DOTAP reagents. It is interesting that an improvement in transfer efficiency was observed in primary cultures transfected with Lipofectin compared with C2C12 cultures transfected with the same liposome formulation (Fig. 6). Despite this improvement with the Lipofectin reagent, the absolute efficiency was still relatively low (3–4%). There was no detectable β -galactosidase gene expression in any of the cultures transfected with the LipofectACE reagent. These results in mouse cells contrast with other reports where much higher efficiencies of gene transfer to rat primary muscle cultures have been reported using the Lipofectin reagent (Jiao et al., 1992a) and to primary hepatocyte cultures using the LipofectACE reagent (Jarnagin et al., 1992).

Recombinant dystrophin gene transfer into *mdx* myoblasts

In a continuation of reporter gene studies in primary culture, the LipofectAMINE and DOTAP reagents were used to transfect myoblasts isolated from *mdx* mice with the dystrophin cDNA plasmid. Samples from transfected *mdx* myoblast cultures were subjected to western analysis, and the resulting immunoblot was probed with 6D3 anti-rod domain dystrophin antibody. Figure 7 clearly demonstrates the expression of recombinant human dystrophin in cultures transfected with the LipofectAMINE reagent (lane 4). No detectable expression of the transgene was observed in the culture transfected with the DOTAP culture.

Direct gene transfer into differentiated myotubes

In the studies described so far proliferating myoblasts were the target cells for liposome-mediated gene transfer. To determine whether postmitotic multinucleate myotubes were also directly susceptible to this form of gene transfer, the optimised protocols for the DOTAP, Lipofectin, and LipofectAMINE reagents were applied to cultures of differentiated myotubes with the pRSVLacZ plasmid. No transgene expression was detected in any primary myotube cultures transfected with DOTAP, Lipofectin, or LipofectAMINE. As shown in Fig. 8 transfected C2C12 myo-

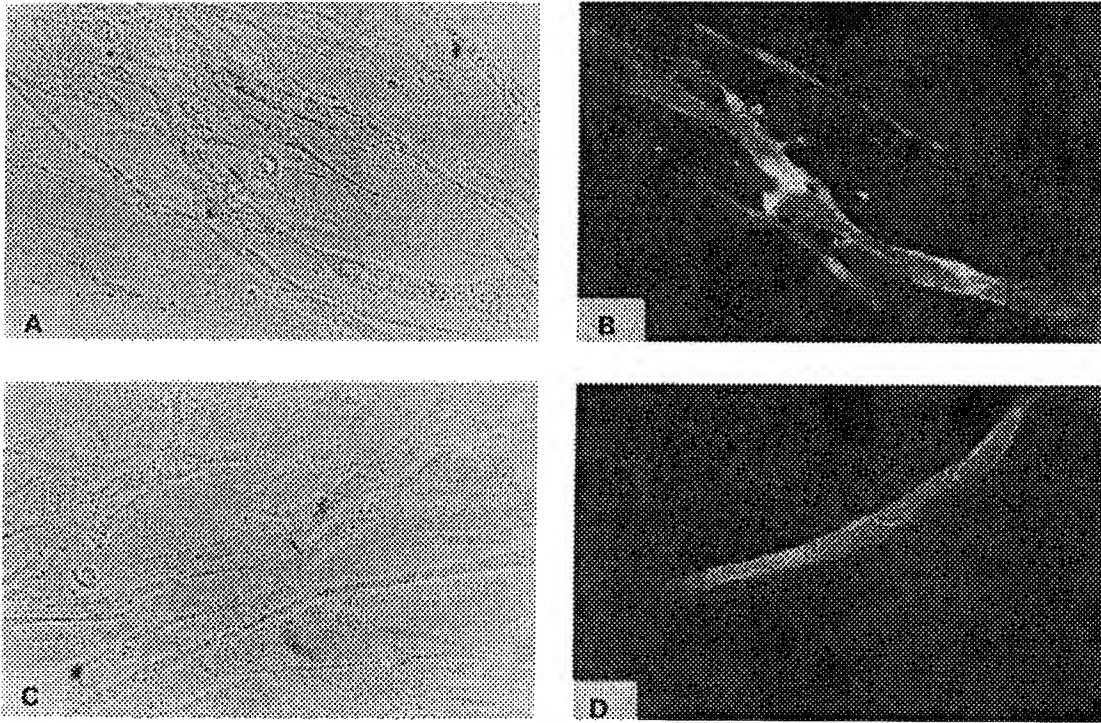


FIG. 4. Human dystrophin expression in murine C2C12 myotubes following optimised transfection. C2C12 myoblasts (1.5×10^3) were plated onto 35-mm-diameter dishes and grown overnight before transfection the following morning with $2 \mu\text{g}$ of pRSVDy complexed with $10 \mu\text{g}$ of LipofectAMINE reagent (A and B) or $8 \mu\text{g}$ of DNA complexed with $8 \mu\text{g}$ of LipofectAMINE reagent (C and D). Medium was replaced with 5% HIHS after 6 h, and cultures were allowed to grow for 10 days before being probed with 12B2 (anti-N-terminal domain dystrophin). B and D are immunofluorescence images showing the expression of the recombinant human dystrophin in a few myotubes among a population of negatively staining myotubes, as seen by the phase-contrast images in A and C. $\times 900$.

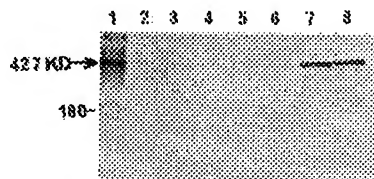


FIG. 5. Immunoblot shows recombinant dystrophin expression in lipofected C2C12 myoblast cultures. C2C12 myoblasts were transfected with either $10 \mu\text{g}$ of DOTAP reagent complexed with 8, 16, or $32 \mu\text{g}$ of DNA (lanes 3–5, respectively) or $10 \mu\text{g}$ of LipofectAMINE reagent complexed to 4, 2, or $1 \mu\text{g}$ of DNA (lanes 6–8, respectively). Cultures were harvested 48 h posttransfection, and the resulting western transfer was probed with 6D3 (anti-rod domain) dystrophin antibody. Expression of the recombinant dystrophin was demonstrated in the normal C57/B10 mouse culture but only detectable in the DOTAP-transfected cultures using the optimal lipid:DNA ratio ($10 \mu\text{g}$ of DOTAP/ $8 \mu\text{g}$ of DNA) (lane 3). In cultures transfected with the LipofectAMINE reagent dystrophin protein was clearly detected in cultures transfected with either 1 or $2 \mu\text{g}$ of DNA complexed with $10 \mu\text{g}$ of LipofectAMINE (lanes 7 and 8, respectively) compared with the untransfected, negative control culture (lane 2). Lane 1, control muscle sample.

tube cultures demonstrated very low expression of the reporter gene only with the DOTAP and LipofectAMINE reagents. This latter observation may be accounted for by low levels of residual myoblast proliferation in these transformed cell cultures.

DISCUSSION

Recently, intense interest has developed in the use of polycationic liposomes for the transfer of recombinant genes into various tissues both in vitro and in vivo (Debs et al., 1987, 1989; Lidgate et al., 1988; Alton et al., 1993; Zhu et al., 1993; Felgner et al., 1994). In the present study, we have demonstrated the use of liposomes for high-efficiency gene transfer into skeletal muscle myoblasts in culture. Unlike viral vector systems, the liposome vehicles are not hindered by size constraints in the foreign DNA to be carried, and transfer of a cDNA-based plasmid gene construct ($>18 \text{ kb}$) encoding full-length recombinant human dystrophin was also obtained.

Dramatic differences were observed among various different liposome formulations, in terms of their abil-

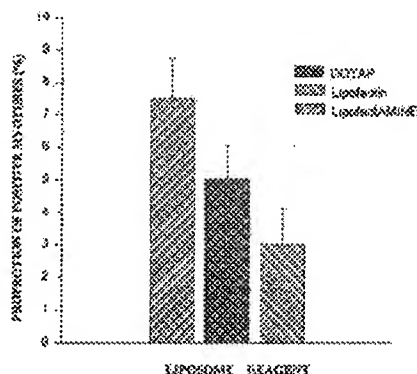


FIG. 6. Maximal efficiency of gene transfer into primary cultures of the C57/B10 mouse. Under previously optimised transfection parameters, 1.5×10^5 primary myoblasts were transfected with $10 \mu\text{g}$ of Lipofectin/ $1 \mu\text{g}$ of DNA (pRSVLacZ), $10 \mu\text{g}$ of LipofectAMINE/ $2 \mu\text{g}$ of DNA, $10 \mu\text{g}$ of LipofectACE/ $1 \mu\text{g}$ of DNA, or $10 \mu\text{g}$ of DOTAP/ $8 \mu\text{g}$ of DNA for 6 h. β -Galactosidase expression was assayed for 5 days posttransfection. Transfection efficiencies of up to 9% were obtained with the LipofectAMINE reagent, up to 5% with the DOTAP reagent, and 4% with the Lipofectin reagent. No reporter gene expression was detected in any of the primary cultures transfected with the LipofectACE reagent.

ity to transfer recombinant genes efficiently into muscle cells. Of the four reagents tested, the LipofectAMINE reagent produced the highest transfection efficiencies in both immortalised cells and primary myoblasts. This property may be due to the higher surface charge concentration on the active polycation component of these vesicles compared with the others examined. However, it is clear that gene transfer mediated by liposome vesicles occurs in a highly specific manner with several variables determining transfection efficiency, of which the liposome concentration and lipid:DNA composition ratio are probably the most crucial. It is thought that complexing between lipid and DNA in these systems occurs such that the liposomes exhibit overall a net positive surface charge, allowing ionic interaction with negatively charged components of the target cell membrane (Stamatatos et al., 1988; Cotten and Wagner, 1993). If addition of DNA indeed neutralises the surface charge of the vesicles, this

FIG. 7. Western blot analysis from *mdx* cultures 48 h post-transfection with pRSVDy, probed with 6D3 (anti-rod domain dystrophin). Lane 1 shows the dystrophin protein band from the C57/B10 mouse positive control culture. Lane 4 demonstrates the expression of full-length recombinant dystrophin in *mdx* cultures following optimised transfection with $10 \mu\text{g}$ of LipofectAMINE reagent and $2 \mu\text{g}$ of pRSVDy. In comparison, the 427-kDa protein band is not detectable in lane 3 (the DOTAP-transfected culture) or in lane 2 (the untransfected, negative control, *mdx* culture).

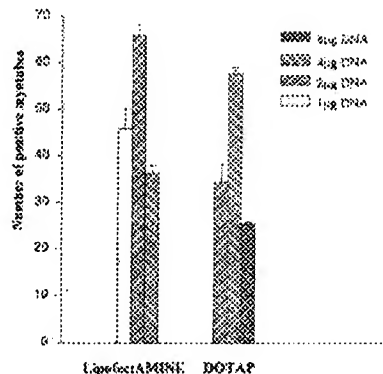
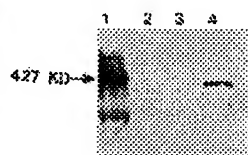


FIG. 8. Expression of the reporter gene following direct myotube transfection. Ten micrograms of LipofectAMINE, DOTAP, or Lipofectin reagents was complexed to the varying amounts of DNA (pRSVLacZ) shown and transfected into 5-day-old C2C12 myotube cultures for 6 h. Cultures were assayed histochemically 3 days later for reporter gene expression. In all the transfected myotube cultures β -galactosidase expression was only poorly detected and was maximal in cultures transfected with the LipofectAMINE reagent using $10 \mu\text{g}$ of reagent and $2 \mu\text{g}$ of DNA.

would explain the decline in transfection efficiency observed when excess DNA is incorporated into the complex.

Our data also suggest that cell division may be critical for efficient gene transfer. In myoblast cultures, plating cell densities that gave high rates of cell division at the time of transfection were also crucial for achieving maximal transfection efficiency. Mitosis may be a requisite to allow exogenous DNA to reach the nuclear transcriptional machinery because during cell proliferation the nuclear envelope is transiently dispersed, allowing access to the cytosolic transgene. Such an effect will not, however, directly influence transgene integration. In a similar way, the differences in transfection efficiencies observed between primary and immortalised muscle cells may reflect in part differing rates of cell division. The observation that post-mitotic myotubes are virtually completely refractory to liposome-mediated gene transfer also supports the view that cell division is crucial. In this respect it may be of some importance to examine cell cycle synchronisation and the use of specific growth factors to maximise overall cell division rates in the interval during or immediately after transfection.

The introduction of recombinant reporter gene and dystrophin cDNA constructs using liposome vesicles now has added skeletal myoblasts to the rapidly evolving list of tissues susceptible to this mode of transfection. The expression of recombinant dystrophin in dystrophin-deficient *mdx* myoblasts following in vitro lipofection forms a basis from which to examine in vivo studies on liposomal gene transfer directly into *mdx* mouse muscle, other animal models of DMD, and indeed many conditions where myoblast stem cell prolif-

eration and myofibre regeneration are ongoing. Second, liposomal gene transfer may be useful for ex vivo gene transfer strategies whereby genetically modified myoblasts or myotubes can be prepared for implantation into either muscle (Barr and Leiden, 1991; Dai et al., 1992) or other ectopic sites, such as in the nervous system (Jiao et al., 1992b), serving as a platform for heterologous transgene expression.

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EXHIBIT J

Circulation

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Gene Therapy for Myocardial Angiogenesis : Initial Clinical Results With Direct Myocardial Injection of phVEGF 165 as Sole Therapy for Myocardial Ischemia

Douglas W. Losordo, Peter R. Vale, James F. Symes, Cheryl H. Dunnington, Darryl D. Esakof, Michael Maysky, Alan B. Ashare, Kishor Lathi and Jeffrey M. Isner

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Gene Therapy for Myocardial Angiogenesis

Initial Clinical Results With Direct Myocardial Injection of phVEGF₁₆₅ as Sole Therapy for Myocardial Ischemia

Douglas W. Losordo, MD; Peter R. Vale, MD; James F. Symes, MD; Cheryl H. Dunnington, MS;
Darryl D. Esakof, MD; Michael Maysky, MD; Alan B. Ashare, MD;
Kishor Lathi, MD; Jeffrey M. Isner, MD

Background—We initiated a phase 1 clinical study to determine the safety and bioactivity of direct myocardial gene transfer of vascular endothelial growth factor (VEGF) as sole therapy for patients with symptomatic myocardial ischemia.

Methods and Results—VEGF gene transfer (GTx) was performed in 5 patients (all male, ages 53 to 71) who had failed conventional therapy; these men had angina (determined by angiographically documented coronary artery disease). Naked plasmid DNA encoding VEGF (phVEGF₁₆₅) was injected directly into the ischemic myocardium via a mini left anterior thoracotomy. Injections caused no changes in heart rate (pre-GTx=75±15/min versus post-GTx=80±16/min, $P=NS$), systolic BP (114±7 versus 118±7 mm Hg, $P=NS$), or diastolic BP (57±2 versus 59±2 mm Hg, $P=NS$). Ventricular arrhythmias were limited to single unifocal premature beats at the moment of injection. Serial ECGs showed no evidence of new myocardial infarction in any patient. Intraoperative blood loss was 0 to 50 cm³, and total chest tube drainage was 110 to 395 cm³. Postoperative cardiac output fell transiently but increased within 24 hours (preanesthesia=4.8±0.4 versus postanesthesia=4.1±0.3 versus 24 hours postoperative=6.3±0.8, $P=0.02$). Time to extubation after closure was 18.4±1.4 minutes; average postoperative hospital stay was 3.8 days. All patients had significant reduction in angina (nitroglycerin [NTG] use=53.9±10.0/wk pre-GTx versus 9.8±6.9/wk post-GTx, $P<0.03$). Postoperative left ventricular ejection fraction (LVEF) was either unchanged ($n=3$) or improved ($n=2$, mean increase in LVEF=5%). Objective evidence of reduced ischemia was documented using dobutamine single photon emission computed tomography (SPECT)-sestamibi imaging in all patients. Coronary angiography showed improved Rentrop score in 5 of 5 patients.

Conclusions—This initial experience with naked gene transfer as sole therapy for myocardial ischemia suggests that direct myocardial injection of naked plasmid DNA, via a minimally invasive chest wall incision, is safe and may lead to reduced symptoms and improved myocardial perfusion in selected patients with chronic myocardial ischemia. (*Circulation*. 1998;98:2800-2804.)

Key Words: angiogenesis ■ ischemia ■ myocardium

Intramuscular transfection of genes encoding angiogenic cytokines¹ may constitute an alternative treatment strategy for patients with severe myocardial ischemia. This strategy is designed to promote the development of supplemental collateral blood vessels that will constitute endogenous bypass conduits around occluded native arteries, a strategy termed “therapeutic angiogenesis.”²

This study describes the initial clinical experience with myocardial gene transfer as sole therapy for refractory angina pectoris. Five patients with chronic, severe angina underwent direct myocardial gene transfer of naked DNA encoding vascular endothelial growth factor (VEGF). There were no operative complications. All patients experienced marked

symptomatic improvement and/or objective evidence of improved myocardial perfusion. This preliminary clinical experience suggests that therapeutic angiogenesis represents a potentially useful strategy for patients with coronary artery disease.

Methods

Patients

Patients were eligible for intramyocardial gene therapy if they had functional class 3 or 4 exertional angina, refractory to maximum medical therapy, areas of viable but underperfused myocardium, and multivessel occlusive coronary artery disease. Subjects were excluded if they had any of the following: a successful revasculariza-

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TABLE 1. Demographic and Clinical Data

Patient	Age	DM	Prior Revascularization	CCS FC		Medications
				Pre-	Post-	
1	67	0	CABG 1988; PTCA 1997	4	1	ASA, BB, N
2	69	+	CABG 1992, 1997	4	2	ASA, BB, CCB, D, N
3	53	0	PTCA 1989; CABG 1991, 1992	4	2	ASA, CCB, N
4	71	0	CABG 1982, 1984; PTCA 1992	4	1	ASA, BB, D, N
5	59	+	CABG 1994; PTCA 1996, 1997	4	2	ACE, ASA, BB, D, N

Pre- and Post- refer, respectively, to status of gene therapy.

ACE indicates angiotensin converting enzyme inhibitor; ASA, aspirin; BB, beta-blocker; CABG, coronary artery bypass graft; CCB, calcium channel blocker; CCS, Canadian Cardiovascular Study; D, diuretic; DM, diabetes; FC, functional class; N, nitrates; PTCA, percutaneous coronary angioplasty including balloon angioplasty, stent, directional and rotational atherectomy.

tion within the previous 6 months, cancer, retinopathy, or an ejection fraction (EF) <20%.

Plasmid DNA (phVEGF₁₆₅)

All patients received eukaryotic expression vector encoding the 165-amino acid isoform of the human VEGF gene (previously described)^{3,4} transcriptionally regulated by the cytomegalovirus promoter/enhancer (phVEGF₁₆₅).^{5,6}

Myocardial phVEGF₁₆₅ Transfer

Plasmid DNA (125 µg) was administered by direct myocardial injection in 4 aliquots of 2.0 mL each via a mini-thoracotomy to the anterolateral left ventricular free wall. Continuous transesophageal echocardiographic monitoring was performed throughout the procedure. Patients were extubated in the operating room and monitored according to the protocol used for minimally invasive CABG.

SPECT Myocardial Perfusion Study

Subjects underwent a dobutamine single photon emission computed tomography (SPECT)-sestamibi study <2 weeks before gene transfer, with the use of dobutamine infusion up to 40 µg · kg⁻¹ · min⁻¹. The acquisition of the poststress SPECT image began 10 minutes after the end of the stress period. Redistribution images were recorded either before or at least 4 hours after stress with the subject at rest. Redistribution and reinjection data were reconstructed in short-axis, vertical, and longitudinal long-axis views for analysis. With the use of the 13-segment model, viability and perfusion scores were assigned to each segment on the basis of the results of the nuclear scan. Perfusion was recorded as normal or abnormal. Segments were visually characterized as fixed, partially reversible, or totally reversible. On days 30 and 60, subjects underwent repeat nuclear perfusion testing using the identical stress protocol and isotope used at baseline.

Coronary Angiography

Patients underwent diagnostic angiography <1 month before and 60 days after gene transfer. All angiograms were interpreted by a reviewer blinded to the patient's name, date of study, and sequence of study (ie, pre- versus posttreatment). Collaterals were graded⁷ as absent (0); filling of side-branches of a target-occluded epicardial coronary artery via collaterals without visualization of the epicardial coronary artery itself (1+); partial filling of the epicardial segment via collateral arteries (2+); and complete filling of the epicardial segment (3+). Each pair of films (baseline and follow-up) was scored independently.

Statistical Analysis

Data are reported as mean±SEM. Comparisons between paired variables were performed using a Student *t* test with a significance level of *P*<0.05.

Results

Patients

Demographic and clinical data for the 5 men (aged 63.8±3.4 years) treated with phVEGF₁₆₅ are shown in Table 1.

Perioperative Course

All patients underwent successful myocardial gene transfer. Mean operative time was 101.6±8.9 minutes. Patients were extubated 18.4±1.4 minutes postoperatively. Injections caused no changes in heart rate (75±15/min versus 80±16/min), systolic blood pressure (114±7 versus 118±7 mm Hg), or diastolic BP (57±2 versus 59±2 mm Hg). Ventricular arrhythmias were limited to unifocal extrasystolic beats (maximum n=5) at the moment of injection. Postoperative Cardiac output fell transiently but increased within 24 hours (preanesthesia=4.8±0.4 versus postanesthesia=4.1±0.3 versus 24 hours postoperative=6.3±0.8, *P*=0.02). Serial ECGs showed no evidence of myocardial infarction in any patient; no patient had an increase in creatine kinase isoenzyme above normal limits. Intraoperative blood loss was 5 to 50 cm³, and total chest tube drainage was 110 to 395 cm³. There were no major perioperative complications. Postoperative LVEF was either unchanged (n=3) or improved (n=2, mean increase in LVEF=5%). All patients were discharged on postoperative day 4 except patient 2 who was discharged on postoperative day 3.

Change in Clinical Status

All 5 patients experienced a decrease in anginal frequency and severity (Table 1). There was no change in the anginal

TABLE 2. Perfusion Scan Results

Patient	Number of Segments								
	Normal Perfusion			Reversible Defect			Fixed Defect		
	Base	D30	D60	Base	D30	D60	Base	D30	D60
1	8	9	9	3	3	3	2	1	1
2	9	9	10	2	4	3	2	0	0
3	5	6	6	5	6	6	3	1	1
4	5	7	8	5	4	3	3	2	2
5	3	6	7	8	5	4	2	2	2

Base indicates baseline; D30, 30-day follow-up study; D60, 60-day follow-up study.

TABLE 3. Angiographic Results

Patient	Extramural Vessel	Rentrop Score	
		Pre-GTx	Post-GTx
1	RCA via LAD/Diag via SVG	1	2
2	OMB via SVG	1	2
	PDA via LAD via LIMA	1	2
3	RCA via LCX	0	3
	Diag via LAD via LIMA	1	3
4	Diag via LAD via LIMA	0	1
	RCA via LCX and Septal	1	2
5	Diag via LAD via LIMA	0	1

RCA indicates right coronary artery; LAD, left anterior descending; Diag, diagonal; SVG, saphenous vein graft; OMB, obtuse marginal branch; PDA, posterior descending artery; LIMA, left internal mammary artery; and LCX, left circumflex.

pattern in any patient up to 10 days post-gene transfer. All patients began to experience a reduction in angina between 10 and 30 days after gene transfer. Angina was completely abolished in 2 patients (patients 1 and 4); patient 5, who has previously experienced daily angina, had only 2 episodes of angina between the day 30 and day 60 follow-up visits. Patients 2 and 3 continued to experience occasional angina but with reduced frequency and at much higher levels of activity. Nitroglycerin (NTG) use for the group of 5 patients decreased from 7.7 ± 1.4 to 1.4 ± 1.0 tablets per day by 60 days post-gene transfer ($P < 0.05$). Brief synopses of the clinical courses of these 5 patients are provided below.

Patient 1, a 67-year-old man, experienced daily angina induced by mild activity requiring an average of 8 tablets

NTG/d. All native vessels and 3 of 4 bypass grafts were occluded. Several institutions had advised the patient that the small caliber of his remaining native vessels precluded repeat CABG. Beginning 21 days after gene transfer, the patient experienced a decrease in the frequency and severity of his angina. By postoperative day 60, the patient was no longer experiencing angina and was no longer requiring NTG. He was able to engage in activities, such as swimming, which were previously impossible because of anginal pain.

Patient 2, a 69-year-old man, experienced daily angina precipitated by activity such as walking 10 yards; for several months he had been taking 12 tablets NTG/d. A vein graft to the left obtuse marginal (LOM) was occluded, and a diffusely diseased vein graft to a diagonal branch of the left anterior descending (LAD) coronary artery was not amenable to percutaneous revascularization. Additional surgery was not feasible because of poor target vessels. For 3 weeks after gene transfer, his symptoms remained unchanged. The patient then began to notice a decrease in NTG consumption accompanied by the ability to increase his level of activity. By day 60, the patient was able to exercise on the bicycle at his local gymnasium for up to 30 minutes. The patient's NTG requirement decreased to a maximum of 2 tablets/d for occasional episodes of mild angina.

Patient 3, a 53-year-old man, experienced daily angina induced by walking ≤ 50 yards and used 6 NTG tablets/d. All native vessels were occluded; grafts to the LAD and right coronary artery (RCA) were patent, whereas an LOM graft was occluded. Percutaneous revascularization was not possible and a third bypass operation for single vessel bypass to a small-caliber target vessel was not feasible. The patient

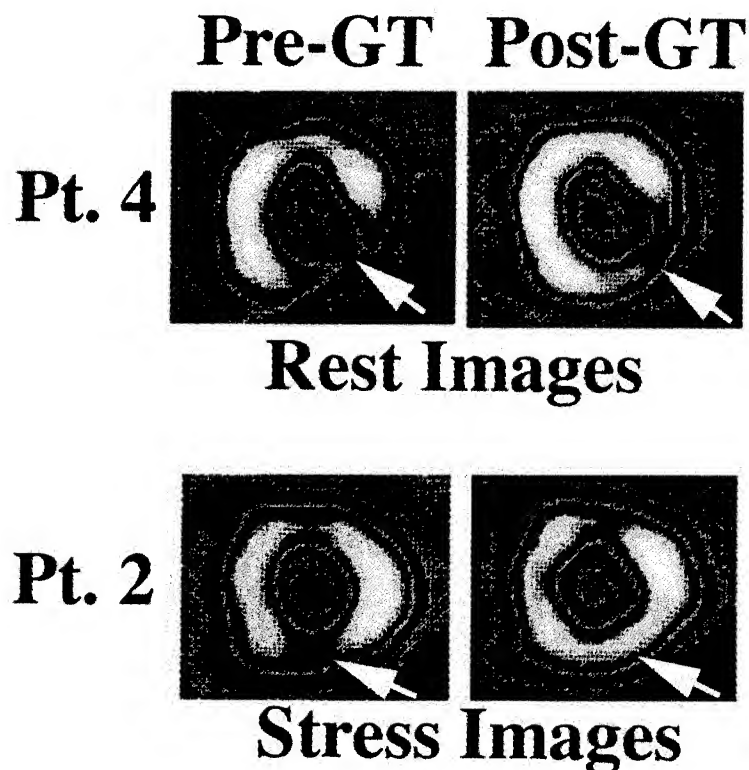


Figure 1. SPECT-sestamibi perfusion imaging. Top, Example of improvement in a "fixed" defect (perfusion abnormality on resting image). In patient (Pt.) 4, a moderate area of decreased perfusion is seen in the infero-lateral wall (arrow) before gene therapy. After gene therapy, perfusion is improved. Bottom, Example of improvement in an area of ischemia. In Pt. 2, a small zone of decreased perfusion is seen in the inferior wall (arrow) before treatment. After treatment, the matching scan shows no evidence of this perfusion defect while a zone of ischemia on the anterior (opposite) wall persists.

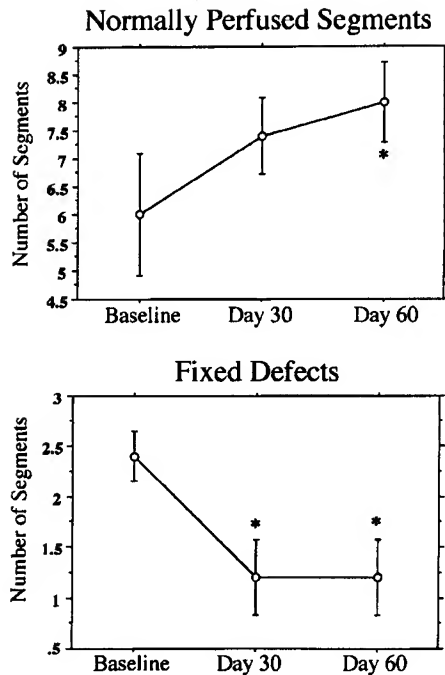


Figure 2. SPECT-sestamibi perfusion imaging: summary of findings in 5 patients. Short-axis views were divided into a total of 13 segments and graded as normal (no perfusion defect), reversible (perfusion defect during stress that partially or completely reversed at rest), or fixed (perfusion defect during stress that persists at rest). Values represent mean \pm SEM for all 5 patients at baseline, 30 days, and 60 days post-gene therapy. * $P < 0.05$ compared with baseline.

experienced no change in anginal symptoms until postoperative week 2, when he noticed an increase in the level of activity required to induce angina. At that time, he was able to perform activities (eg, planting in his garden) that he had not previously engaged in for several months; NTG use decreased to 5 tablets per week. By 60 days after gene transfer, he was able to walk up to one-half of a mile without experiencing angina.

Patient 4, a 71-year-old man, complained of daily angina precipitated by walking < 100 yards. All native vessels and grafts to the RCA and LOM were occluded. Percutaneous revascularization was not possible and repeat surgery was not feasible because of small-caliber target vessels. Beginning on postoperative day 10, the patient noted increased exercise capacity accompanied by decreased NTG use. By day 30 follow-up, the patient was requiring no NTG and had returned to his 5 hour per day position doing maintenance for his church. Between days 30 and 60, the patient developed dyspnea, associated with inadvertent discontinuation of his daily diuretic (furosemide, 80 mg). After resumption of his diuretic, his symptoms resolved and he resumed his increased activity level without anginal symptoms, dyspnea, or NTG use.

Patient 5, a 59-year-old man with daily angina precipitated by walking 10 to 20 yards, also required continuous oxygen because of severe chronic obstructive pulmonary disease. He had been recently hospitalized for several months because of intractable angina requiring intravenous NTG. All native vessels and grafts to RCA and diagonal branch of the LAD

were occluded. Percutaneous revascularization was not possible and a third bypass operation was not feasible because of poor distal vessels. By postoperative day 30, the patient noted that he was experiencing no angina and was able to walk distances of up to 500 yards. Additionally, he found that his use of supplemental oxygen had decreased. At day 60 follow-up, he reported a total of 2 anginal episodes in the previous month, each of which was resolved with a single NTG tablet.

SPECT-Sestamibi Perfusion Imaging

All patients had improvement in myocardial perfusion, revealed by comparison between pre- and posttreatment (Figure 1) SPECT-sestamibi imaging (Table 2). The mean number of normally perfused segments per patient increased from 6.0 ± 1.1 before gene transfer to 8.0 ± 0.7 ($P < 0.05$) at day 60 after gene transfer (Figure 2). This was accompanied by a decrease in the mean number of irreversibly ischemic segments from 2.4 ± 0.2 to 1.2 ± 0.4 ($P < 0.05$) at day 60 follow-up examination (Figure 2).

Coronary Angiography

Selective coronary angiography was performed before and 59.8 ± 1.5 days after gene transfer (Table 3). Angiographic evidence for improved collateral flow into ischemic areas of the myocardium was observed in all 5 patients. The evidence of new collateral vessels consisted of improved filling of 4 previously identified vessels as well as the development of collaterals to 3 vessels which previously had no collateral filling. In 2 patients, there was improvement by a single Rentrop grade in one vessel territory; the other 3 patients demonstrated improvement in 2 territories by 1 to 3 Rentrop grades.

Discussion

The finding that VEGF could be used to achieve angiogenesis that was therapeutic was first demonstrated by Takeshita et al,² who administered rhVEGF as a single intra-arterial bolus to rabbits with unilateral hindlimb ischemia. Similar findings with rhVEGF administration in canine⁸ and porcine⁹ models of myocardial ischemia were published shortly thereafter.

Gene transfer constitutes an alternative strategy for accomplishing therapeutic angiogenesis in patients with limb and myocardial ischemia. In VEGF, this is a particularly appealing strategy because the VEGF gene encodes a signal sequence which permits the protein to be naturally secreted from intact cells.⁴ Previous studies from our laboratory^{10,11} indicated that arterial gene transfer of cDNA encoding for a secreted protein could yield meaningful biological outcomes despite a low transfection efficiency. Indeed, preclinical animal studies established the feasibility of achieving therapeutic angiogenesis after site-specific gene transfer of naked DNA encoding VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉.¹² Subsequent clinical experience documented histological and angiographic evidence of phVEGF₁₆₅-induced neovascularization in patients with critical limb ischemia.^{5,6} These findings established proof of principle for the concept that the angiogenic activity of VEGF is sufficiently potent to achieve therapeutic benefit.

The present study provides the first evidence for a favorable clinical effect of direct myocardial injection of naked plasmid DNA encoding for VEGF. Each patient experienced a reduction in anginal symptoms and nitrate use, and there is objective evidence for reduced ischemia by perfusion imaging. Because each patient enrolled in this study had long-standing, stable, severe angina, the change in clinical status observed for these 5 patients is unlikely to represent random chance. In contrast to work recently reported by Schumacher et al,¹³ in which administration of fibroblast growth factor-1 (FGF-1) was combined with conventional surgical revascularization,¹³ the present study used VEGF gene transfer as the sole therapeutic intervention.

This early experience, although encouraging from the standpoint of therapeutic angiogenesis and gene therapy, leaves several issues unresolved. Optimizing the anatomic site, number, and dose of intramyocardial injections will require further investigation. The FDA, Recombinant Advisory Committee of the NIH, and St. Elizabeth Medical Center Human Investigation Research and Institutional Biosafety Committees all concurred that the strategy of gene therapy alone administered via a mini-thoracotomy would not permit randomization against placebo (untreated controls). We anticipate that incorporation of a placebo group and clinical testing of alternative dosing regimens, including multiple treatments, will be addressed on availability of a catheter-based system for reliable percutaneous myocardial gene delivery; this is currently under preclinical investigation.¹⁴

Furthermore, the choice of appropriate formulation or vector in the case of VEGF remains to be determined. As indicated above, rhVEGF protein has been shown to be efficacious for treatment of limb and myocardial ischemia in preclinical studies, and preliminary clinical investigation of rhVEGF¹⁵ together with the aforementioned studies of Schumacher et al have suggested the potential usefulness of recombinant protein for therapeutic angiogenesis. The use of an adenoviral vector expressing VEGF₁₂₁ has been shown to improve myocardial perfusion and function in a swine model of myocardial ischemia¹⁶ and is now being tested in human subjects. Likewise, alternatives to VEGF, including FGF-1,¹⁷ FGF-2,¹⁸ and FGF-5¹⁹ are or will be investigated as genes or recombinant proteins in clinical trials of therapeutic angiogenesis.

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EXHIBIT K

Intramyocardial Gene Therapy with Naked DNA Encoding Vascular Endothelial Growth Factor Improves Collateral Flow to Ischemic Myocardium*

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ABSTRACT

Both VEGF protein and VEGF DNA in combination with an adenoviral vector have been shown to enhance collateral formation in a porcine model of chronic myocardial ischemia. We sought to determine whether direct intramyocardial injection of naked DNA encoding for VEGF could similarly improve myocardial perfusion. Initially, 23 nonischemic pigs received either 200 μ g of plasmid DNA encoding β -galactosidase (pCMV β , $n = 11$) or 500 μ g of phVEGF165 ($n = 12$) into four separate sites in the myocardium via a small anterolateral thoracotomy incision in the fourth intercostal space. Two additional groups of pigs received an intramyocardial injection of either phVEGF165 ($n = 6$) or pCMV β ($n = 7$) 3 to 4 weeks after implantation of an ameroid constrictor around the left circumflex coronary artery. The injections caused no change in heart rate or blood pressure, and no ventricular arrhythmias or histologic evidence of inflammation. VEGF protein was detected by Western blot in VEGF-treated animals, with the strongest bands closest to the injection site. Plasma VEGF concentration (ELISA) increased from 3 ± 2 to 27 ± 13 pg/ml ($p = 0.035$) by day 4 after treatment. No increase in VEGF protein was noted in pCMV β -treated animals whereas these did stain positive for β -Gal. Resting myocardial blood flow (colored microspheres) was significantly reduced in the ischemic versus nonischemic territory in control animals (1.07 ± 0.05 versus 1.32 ± 0.05 ; $p < 0.05$) but not VEGF-treated pigs (1.32 ± 0.24 versus 1.13 ± 0.12 ; $p = \text{NS}$). Maximal vasodilatation with adenosine significantly increased flow to the ischemic region in VEGF-treated pigs (2.16 ± 0.57 versus 1.32 ± 0.24 ; $p < 0.05$) but not controls (1.31 ± 0.05 versus 1.17 ± 0.06 ; $p = \text{NS}$). Collateral filling of the occluded circumflex artery improved in five of six VEGF-treated pigs (mean change in Rentrop score, +1.5). We conclude that direct intramyocardial transfection phVEGF165 is safe and capable of producing sufficient VEGF protein to enhance collateral formation and myocardial perfusion. This approach may offer an alternative therapy for patients with intractable myocardial ischemia not amenable to PTCA or CABG.

OVERVIEW SUMMARY

Recombinant vascular endothelial growth factor protein and its gene combined with an adenoviral vector are capable of stimulating collateral formation in animal models of chronic myocardial ischemia. To determine whether direct intramyocardial injection of VEGF DNA without a viral vector could be performed safely and similarly improve myocardial perfusion, we injected plasmid DNA encoding ei-

ther β -galactosidase (controls) or VEGF₁₆₅ directly into the myocardium of pigs who had previously had an ameroid constrictor implanted around the circumflex coronary artery. The injections caused no significant deleterious effects to the animals and VEGF protein was detected both in the myocardial tissue and in the plasma of the animals treated with VEGF₁₆₅. Myocardial blood flow measured by colored microspheres was significantly improved in the ischemic territory of VEGF-treated animals but not con-

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trols. Coronary angiography demonstrated improved collateral filling of the occluded circumflex artery in five of six VEGF-treated pigs but in only one of the control animals. We conclude from this study that direct intramyocardial administration of VEGF₁₆₅ can be performed safely and is capable of producing sufficient VEGF protein to enhance collateral development and improve myocardial perfusion.

INTRODUCTION

THE TREATMENT of chronic stable angina is aimed at maintaining a positive balance between myocardial oxygen supply and demand. The first-line treatment in most patients will be pharmacologic therapy, followed by angioplasty or bypass surgery. In some patients who have diffuse coronary artery disease, or have had multiple previous interventions, repeat angioplasty or coronary artery bypass graft (CABG) may not be feasible because of poor target vessels, lack of conduits, or unacceptable operative risk. Despite maximal pharmacological therapy many of these "inoperable" patients are incapacitated by frequent anginal attacks.

Vascular endothelial growth factor (VEGF) is a potent angiogenic mitogen. Administration of the protein itself and transfection with VEGF DNA have been shown to improve collateral blood flow to ischemic limbs both in animal models and, in the case of VEGF DNA, in a phase I clinical trial (Takeshita *et al.*, 1994a; Tsurumi *et al.*, 1996; Baumgartner *et al.*, 1998). The present study was conducted, first, to evaluate the feasibility and optimal technique of intramyocardial gene transfer, and second, to assess the efficacy and safety of direct intramyocardial gene therapy with VEGF in a porcine model of myocardial ischemia.

MATERIALS AND METHODS

Animal model

Thirty-six Yorkshire swine weighing 25–35 kg were studied under protocols approved by the Animal Care and Use Committee of St. Elizabeth's Medical Center (Boston, MA) and in accordance with the *Guide for the Care and Use of Laboratory Animals* [Department of Health and Human Services, publication no. (NIH)86-23, revised 1985].

Animals were retained on a normal diet. All procedures were performed under anesthesia and using sterile techniques. Pigs were anesthetized with an intramuscular injection of ketamine (25 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA), acepromazine (0.2 mg/kg; Fermenta Animal Health Company, Kansas City, MO), and atropine (0.05 mg/kg; Fujisawa, Deerfield, IL). Ventilation with 2% isoflurane (Fort Dodge Laboratories) was used to ensure adequate anesthesia throughout the experiment. Pancuronium (0.08 mg/kg, administered intravenously; Elkins-Sinn, Cherry Hill, NJ) was given as a muscle relaxant.

Levodromaron (2 mg, subcutaneous; Roche Laboratories, Nutley, NJ) was given as analgesic at the beginning of the procedure and supplemented as necessary. Lidocaine (50 mg; Abbott Laboratories, North Chicago, IL) was given prophylactically. On completion of each procedure animals received an

additional injection of levodromaron, and were observed during recovery until fully conscious. All animals received 500 mg of cefazolin (Kefzol; Eli Lilly International, Indianapolis, IN) subcutaneously at the beginning of the procedure and two 500-mg doses for 5 days postoperatively.

At the end of the study period the animals were again anesthetized in the manner described above. Hemodynamic parameters and left ventricular function were evaluated as indicated below. Afterward the animals were killed with euthanasia solution, and the heart, and part of the lungs, liver, and mediastinal lymph nodes, were excised for further analysis.

Study groups

The experimental protocol, including a breakdown of study groups, time of sacrifice, and the number of animals in each section, is illustrated in Table 1.

Nonischemic pigs. Twenty-three pigs were treated with either pCMV β ($n = 11$) or phVEGF₁₆₅ ($n = 12$) and sacrificed after 3–5 days (pCMV β) or 1–8 weeks (phVEGF).

After stable anesthesia was achieved as described above, the left carotid artery was cannulated with a 7F sheath for blood pressure monitoring and catheterization. The pigs were placed in a lateral position, to enable an incision in the left fourth intercostal space. After opening the pericardium the gene solution was injected directly into the myocardium with a 25-gauge needle. To prevent penetration of the left ventricular (LV) wall the needle was covered with a rubber stopper, leaving only 5 mm of the distal end free for injection. In this way the solution was always injected at the same depth. To be sure that no blood vessel or the LV cavity was reached, a check was performed before each injection to determine if blood could be aspirated. If this was the case the needle was readjusted. During introduction of the needle and injection, the heart rhythm was monitored carefully. With the needle in the myocardium the gene solution was slowly injected under visual guidance to assure that no fluid leaked back along the needle. Injection of the DNA was monitored in several pigs with direct epicardial echocardiography. This demonstrated that injection of a volume of 2 ml resulted in distribution of the injectate over an area of 1–2 cm of adjacent myocardium.

TABLE 1. STUDY GROUPS

	Injection volume (ml)	Sacrifice	Number
Nonischemic pigs			
pCMV β	4 \times 0.5	3–5 days	4
	4 \times 2	3–5 days	4
	4 \times 5	3–5 days	3
phVEGF ₁₆₅	4 \times 2	1 week	5
		4 weeks	5
		8 weeks	2
Ischemic pigs			
pCMV β	4 \times 2	4 weeks after treatment	7
phVEGF ₁₆₅	4 \times 2	4 weeks after treatment	6

Ischemic pigs. In a total of 13 pigs chronic ischemia was induced by placing an ameroid constrictor (Research Instruments, Corvallis, OR) around the left circumflex coronary artery. Three weeks later, pigs underwent repeat thoracotomy and were treated by intramyocardial injection of either phVEGF165 ($n = 6$) or pCMV β ($n = 7$).

Plasmids and injectate volume

A nuclear-specific cytomegalovirus (CMV) promoter–enhancer–driven β -galactosidase-carrying plasmid (pCMV β) was used for evaluation of the appropriate injection volume for the intramyocardial injection method. To establish the optimal volume each pig received four intramyocardial injections containing 50 μ g of plasmid DNA. The injection sites were chosen at the corners of a 1 \times 1 cm square. Various volumes per injection site were used: 0.5 ml (total volume, 2 ml; $n = 4$), 2.0 ml (total volume, 8.0 ml; $n = 4$), and 5 ml (total volume, 20 ml; $n = 3$). The β -galactosidase activity in the myocardium was measured after sacrifice. Treatment with the lower volume (4×0.5 ml) in four pigs resulted in a mean β -galactosidase activity of 1578 ± 566 RLU/mg protein, treatment with 4×2 ml ($n = 4$) resulted in 1328 ± 436 RLU/mg protein, and treatment with 4×5 ml ($n = 3$) resulted in 1366 ± 373 RLU/mg protein, all significantly higher than untreated control hearts ($n = 4$; 660 ± 103 RLU/mg). On the basis of these experiments a volume of 4×2.0 ml was chosen for treatment with the plasmid containing DNA for VEGF (phVEGF165). For evaluation of protein production blood samples were taken before and after treatment to measure plasma VEGF protein levels with a commercially available ELISA kit (R&D Systems, Minneapolis, MN). After treatment with the plasmid, pigs were sacrificed after either 1 week ($n = 5$), 4 weeks ($n = 5$), or 8 weeks ($n = 2$).

Evaluation of gene expression

In the pCMV β -treated pigs the β -galactosidase activity in the myocardial tissue was evaluated with a chemiluminescence assay (Tropix, Bedford, MA). Before measuring β -galactosidase activity, tissue homogenates were pretreated with Chelex 100 to inactivate a natural inhibitor of the enzyme (Oswald *et al.*, 1997).

In the phVEGF165-treated pigs gene expression of the plasmid DNA was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) Western blotting for VEGF protein as previously described. In short, tissue specimens were stored at -80°C until assayed. The tissue was homogenized in lysis buffer spun down twice at high speed. Equal amounts (100 μ g) of protein were loaded on a 12% gel under nonreducing conditions. After blotting, the membrane was exposed to a rabbit anti-VEGF antibody (sc152; Santa Cruz Biotechnology, Santa Cruz, CA) followed by the secondary (anti-rabbit) antibody labeled with horseradish peroxidase. Last, the membrane was put in enhanced chemiluminescence (ECL) solution and exposed to X-ray film.

Study parameters

Hemodynamics. Throughout the experiments, the condition of the animals was carefully monitored by means of continu-

ous transcutaneous oximetry, intraarterial blood pressure measurement, and electrocardiographic registration of the limb leads and one modified precordial lead.

Myocardial enzymes. Myocardial enzymes were measured from serum samples at the beginning of the procedure, at the end of the procedure, and the following day for creatine phosphokinase (CPK), and CPK-MB isoenzyme determination.

Left ventricular function. To assess the effect of the injectate volume and procedure on LV function, left ventricular angiograms were obtained at baseline (before thoracotomy) and at the end of the study period, in the nonischemic pigs. Angiograms (6 frames per second) were taken from the 55° left anterior oblique view and from the 35° right anterior oblique view. A planimetric program (MACmeasure 1.9) was used to calculate from these two views left ventricular ejection fractions from end systolic and end diastolic frames. The average of these two measurements was taken for each time point.

Histologic evaluation. The heart of each animal was removed, examined for gross pathologic changes, weighed, and rinsed in cold phosphate-buffered saline (PBS). The left ventricle was dissected free and sliced transversally, perpendicular to the long axis of the heart, into slices 1 cm in thickness at five different levels from the apex to the base of the heart. From each slice sections were taken from anterior, lateral, posterior, and septal regions. In addition, biopsies from right ventricle, right atrium, left atrium, lung, liver, mediastinal lymph nodes, and pericardium were taken.

At all levels one slide was stained with hematoxylin–eosin for evaluation of infiltrative cells.

From all tissues one part was snap-frozen and stored at -80°C for quantitative assay of β -galactosidase, or for Western blotting in the case of phVEGF165 treatment. The other part was fixed in 4% formaldehyde in PBS for 2 hr at 4°C . The tissues were then divided into two parts again. One part was incubated in 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) solution for 20 hr and location of β -galactosidase activity was determined as previously described (Couffignal *et al.*, 1997).

Myocardial perfusion. Myocardial blood flow was determined as described previously (Hariawala *et al.*, 1996). In short, colored microspheres (Triton Technology, San Diego, CA) were used to determine myocardial blood flow in ischemic (circumflex territory) and nonischemic tissue (left anterior descending [LAD] territory). Tissue was taken at two levels—proximally (near the base of the heart) and distally (closer to the apex). Flow was determined at rest and after intravenous administration of 6 mg of adenosine. Microspheres (15 ± 0.1 μ m in diameter) with two different colors (yellow and blue, or yellow and red) were used. Flow was quantified by measuring the dye content of the tissue with a spectrophotometer (HP8452; Hewlett Packard, Palo Alto, CA). Reference blood samples were taken from the femoral artery at a rate of 6 mg/min. Flow was calculated in milliliters per minute per gram of tissue.

Coronary angiography. Via an arterial sheath in the femoral or carotid artery, a 6 or 7F left Judkins coronary diagnostic

catheter was advanced to the ostium of the left coronary artery. Cineangiograms (6 frames per second) were made from the 30° left anterior oblique position, after injection of 500 μ g of nitroglycerin into the left main stem. Contrast medium (10 ml) was injected with a power infusion pump (Medrad Technologies, Philadelphia, PA) at a rate of 3 ml/sec. These angiograms were repeated from the same angles, using the same injection protocol at follow-up. Collateral filling of the occluded circumflex (Cx) artery and its branches was scored according to Rentrop: 0, no filling; 1, filling of small side branches; 2, partial filling of epicardial segments of the circumflex or obtuse marginal (OM) branches; 3, complete filling of epicardial CX or OM branches (Rentrop *et al.*, 1998). Angiograms were presented to a panel of eight observers blinded to therapy and pre- or posttreatment information. The consensus score for each angiogram was taken to evaluate changes in Rentrop score.

RESULTS

Safety issues

The intramyocardial injections of pCMV β did not cause changes in heart rate regardless of the volume injected: 4×0.5 ml (preinjection, 145 ± 23 bpm; postinjection, 127 ± 20 bpm); 4×2 ml (preinjection, 143 ± 4 bpm; postinjection, 142 ± 4 bpm); 4×5 ml (preinjection, 143 ± 5 bpm; postinjection, 145 ± 3 bpm). Diastolic blood pressure did not change after injection of 4×0.5 ml (preinjection, 45 ± 4 mmHg; postinjection, 47 ± 4 mmHg), or after 4×2 ml (pre, 42 ± 2 mmHg; post, 43 ± 2 mmHg), or after 4×5 ml (pre, 44 ± 2 mmHg; post, 44 ± 2 mmHg). The same was found for the systolic blood pressure: 4×0.5 ml (pre, 83 ± 4 mmHg; post, 82 ± 4 mmHg), 4×2 ml (pre, 81 ± 3 mmHg; post, 81 ± 2 mmHg). In the pigs treated with phVEGF165 (4×2 ml), the heart rate did not change (pre, 136 ± 8 bpm; post, 141 ± 5 bpm). Systolic blood pressure decreased slightly (pre, 95 ± 3 mmHg; post, 89 ± 3 mmHg; $p = 0.01$) as did the diastolic blood pressure (pre, 62 ± 3 mmHg; post, 54 ± 2 mmHg; $p = 0.007$). This recovered within 5 min, without major hemodynamic consequences. During injection no malignant ventricular arrhythmias were encountered in any animal. In three pigs nonsustained ventricular tachycardia without hemodynamic deterioration occurred during injection. In the majority of the pigs sporadic premature ventricular contractions (PVCs) were present at the moment of entering the myocardium with the needle. The electrocardiogram did not show evidence of myocardial injury in any of the pigs. In the phVEGF165-treated pigs the CPK level increased from 381 ± 55 IU/liter at the beginning of the procedure, to 766 ± 125 IU/liter at the end of the procedure, and to 5274 ± 1245 IU/liter after 1 day. The myocardial-specific MB isoenzyme, however, did not increase (1.5 ± 0.2 , 2.2 ± 0.5 , and 2.2 ± 0.4 ng/ml). None of the pigs had an MB fraction higher than the normal limit (5.0 ng/ml). The heart weights at sacrifice were determined to detect evidence of myocardial interstitial, but were not different between the groups: pCMV β injections of 4×0.5 ml (6.56 ± 0.73 g/kg), 4×2 ml (5.64 ± 0.29 g/kg), and 4×5 ml (5.84 ± 0.26 g/kg); phVEGF-injected pigs sacrificed after 1 week (6.07 ± 0.32 g/kg), 4 weeks (5.70 ± 0.84 g/kg), and 8 weeks (5.96 ± 0.30 g/kg). On histologic examination no more

than five inflammatory cells per high-power field were present. No arteriovenous anomalies were found in the heart or in other tissues (Fig. 1).

Myocardial and systemic VEGF production

Plasma samples for VEGF protein measurements were taken on a daily basis during the first week and again at 2 and 3 weeks after injection in all pigs treated with phVEGF165. In individual pigs a pronounced increase in VEGF protein concentration was noted. Although individual VEGF increases were variable, the mean VEGF concentration increased from 3.0 ± 2.0 to 27.4 ± 10.9 pg/ml ($p = 0.041$) (Fig. 2). No detectable levels were found in pCMV β -treated pigs. In ischemic pigs the pretreatment VEGF concentrations were 18.6 ± 2.6 and 18.5 ± 6.7 pg/ml in the control and phVEGF groups, respectively. In both groups an increase was found after intramyocardial injection to 50.2 ± 14.3 and 40.1 ± 7.8 pg/ml ($p = \text{NS}$), respectively.

Western blotting of tissue obtained from nonischemic pigs sacrificed 1 week after intramyocardial VEGF gene transfer showed strong bands in the region closest to the injection site (Fig. 3). In the hearts from pigs sacrificed 4 and 8 weeks after treatment fainter but clear bands were still present. No such bands were found in pigs injected with pCMV β . Similar evidence of VEGF protein production was also detected on Western blots of myocardial tissue from all VEGF-treated pigs in the ischemic group.

Ventricular function

Global left ventricular ejection fraction (LVEF) was assessed by ventriculography in VEGF-treated pigs in the nonischemic



FIG. 1. Histologic section taken from the anterior wall of the left ventricle of a pig treated with phVEGF165, showing no evidence of inflammatory reaction or abnormal vascular development.

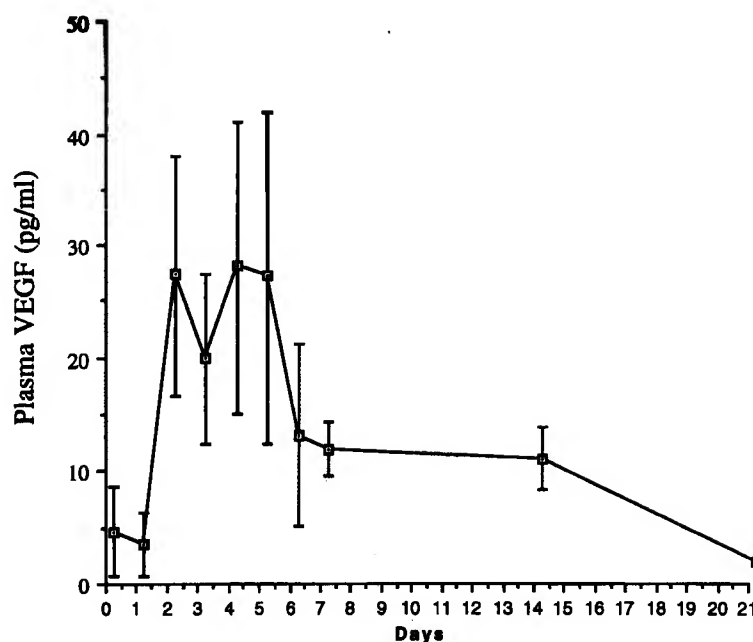


FIG. 2. The plasma concentrations of VEGF protein (ELISA) after treatment with phVEGF165, showing peak levels between day 2 and day 5 after intramyocardial injection. VEGF was no longer detectable in the plasma of any pig after 2 weeks.

group only to look for evidence of any adverse effects of the injection procedures. Mean LVEF at baseline prior to VEGF injection was $39 \pm 6\%$ ($n = 10$) compared with a mean value of $32 \pm 6\%$ at sacrifice in pigs monitored for 1 week ($n = 5$), $46 \pm 6\%$ at 4 weeks ($n = 5$), and $39 \pm 8\%$ at 8 weeks ($n = 2$). No significant difference was detected between any of the post-injection values and the baseline LVEF.

Myocardial blood flow

Myocardial blood flow was measured by the colored microsphere method at rest and after intravenous administration of 6 mg of adenosine (maximal vasodilation) in the ischemic pigs only (Table 2). Resting flow to ischemic myocardium was lower than flow to nonischemic myocardium in the control group but not in the phVEGF165-treated pigs. After maximal vasodilation flow to the nonischemic myocardium increased in

both groups. No increase in flow to ischemic myocardium was found in the control group, whereas in the phVEGF165-treated pigs a significant increase in flow to ischemic myocardium was noted. The maximal flow was lower than the maximal flow to nonischemic myocardium in both groups.

Coronary angiography

Collateral filling of the occluded circumflex artery was evaluated by angiography. On the basis of a blinded comparison of the angiograms from each animal obtained 3 weeks after constrictor placement (at time of gene transfer) with those done 4 weeks after treatment, a collateral score according to Rentrop was determined. In all but two pigs in the control group, a lower Rentrop score was achieved 4 weeks after therapy compared with 3 weeks after constrictor placement. In the phVEGF165-treated animals collateral flow improved in all but one animal as reflected in a higher Rentrop score at follow-up (Fig. 4). The overall change in Rentrop score was significantly greater in the phVEGF165 group (Fig. 5).

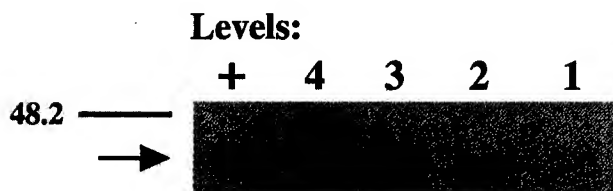


FIG. 3. Western blot of tissue extracts from nonischemic pigs sacrificed 1 week after intramyocardial VEGF gene transfer, showing strong bands in the region closest to the injection site (4) and weaker but demonstrable VEGF protein in sections taken further from the injection site (level 3, 1 cm; level 2, 2 cm; level 1, 3 cm from injection site).

DISCUSSION

VEGF has been shown to be a potent endothelial cell mitogen *in vitro*, with the capability of stimulating neovascularization *in vivo*. The VEGF₁₆₅ protein has been demonstrated to induce collateral formation and augment perfusion in ischemia limbs, as well as in ischemic myocardium (Takeshita *et al.*, 1994b; Harada *et al.*, 1996). Transfection with plasmid DNA encoding VEGF₁₆₅ has been shown to be effective in ischemic limbs in various experimental studies (Takeshita *et al.*, 1996;

TABLE 2. MYOCARDIAL BLOOD FLOW^a

	Nonischemic zone		Ischemic zone	
	Pre	Post	Pre	Post
Control (pCMV β)				
Proximal	1.32 \pm 0.05	1.78 \pm 0.04 ^b	1.07 \pm 0.05 ^c	1.01 \pm 0.06
Distal	1.35 \pm 0.02	1.78 \pm 0.06 ^b	1.17 \pm 0.06 ^c	1.31 \pm 0.053
phVEGF165				
Proximal	1.25 \pm 0.11	1.96 \pm 0.02 ^b	0.96 \pm 0.16	1.37 \pm 0.14 ^{b,c}
Distal	1.13 \pm 0.12	1.67 \pm 0.05 ^b	1.32 \pm 0.24	2.16 \pm 0.57 ^b

^aMyocardial blood flow (ml/g/min) as measured with colored microspheres at baseline (pre) and after 6 mg of adenosine was administered intravenously (Post). Nonischemic zone, LAD territory; ischemic zone, circumflex territory. Proximal samples near base of heart, distal near apex.

^b $p < 0.05$ versus Pre.

^c $p < 0.05$ versus nonischemic value.

Tsurumi *et al.*, 1996). It has also been shown that this treatment strategy is effective in certain patients with end-stage critical limb ischemia (Baumgartner *et al.*, 1998). To extend this form of therapy to patients with inoperable coronary artery disease (CAD) an acceptable method of administering the plasmid to the ischemic myocardium is required. We considered that in patients with advanced CAD, intractable but stable angina, and no other revascularization option, a small thoracotomy might be considered acceptable for this purpose. Furthermore, after plasmid injection, the production of the protein, accompanied by a beneficial effect, should be demonstrated. We performed this study to investigate the feasibility and efficacy of intramyocardial gene transfer with phVEGF165.

Initially the effect of differences in injection volume were considered using pCMV β . We have shown in a previous study that increasing injection volumes from 50 to 300 μ l in rabbit hearts caused increasing transfection efficiency (Gal *et al.*, 1993). Theoretically, increasing injection volumes may facilitate the spread of the DNA into the tissue. However, in the present study, we did not detect a difference in β -galactosidase (β -Gal) activity between injection volumes of 0.5, 2, and 5 ml. Indeed, with the highest volume, a slight decrease in β -Gal activity was observed.

Previous studies investigating experimental angiogenic strategies have utilized either the recombinant protein or DNA plus an adenoviral vector. When the recombinant protein was used it was administered either into the heart as a single injection (Hariawala *et al.*, 1996) or by long-term infusion (Banai *et al.*, 1994). Systemic side effects such as hypotension were of concern after intracoronary administration (Horowitz *et al.*, 1997) but were avoided by longer term infusion and were not seen after intramyocardial injection of phVEGF165 in the present study. The feasibility of adenovirus-mediated gene transfer with angiogenic factors has been demonstrated by a number of studies (Giordana *et al.*, 1996; Mack *et al.*, 1998). The use of adenovirus-mediated gene therapy has a major potential limitation: it may elicit a cellular immune response to viral or transgene antigens resulting in destruction of transfected cells. Therefore, repeated administration of the recombinant virus is typically ineffective. Such immunologic problems can be avoided by using plasmid-mediated transfection.

We previously documented the feasibility and efficacy of plasmid-mediated gene transfer in nonischemic hearts of rabbits and microswine (Gal *et al.*, 1993). The present report is the first to describe the use of nonviral, plasmid-based gene transfer to induce angiogenesis in a model of chronic myocardial is-

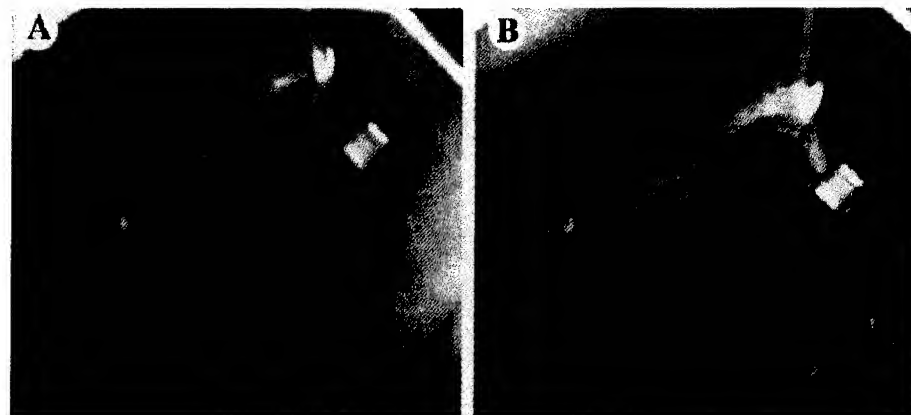


FIG. 4. Left coronary angiograms taken from an ischemic pig treated with phVEGF165 (A) 3 weeks after constrictor placement at the time of VEGF administration and (B) 4 weeks after VEGF administration. Rentrop grade III filling of the distal circumflex vessel by ipsilateral collaterals as well as collaterals from the LAD and its branches is evident.

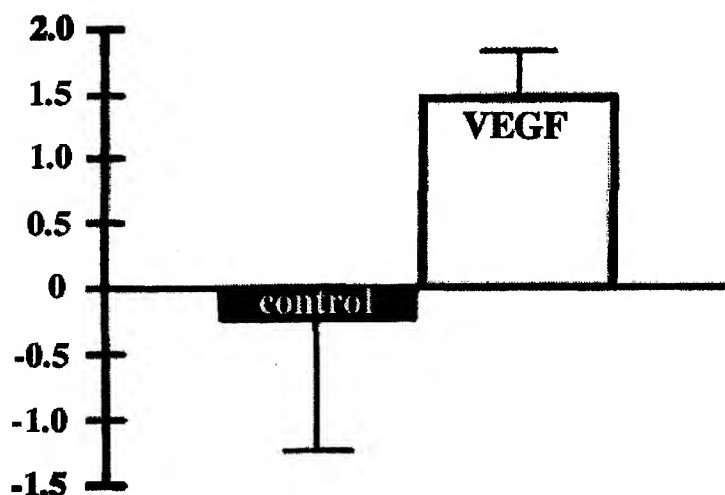


FIG. 5. Change in Rentrop score average for the ischemic pigs in each of the control (pCMV β) and phVEGF165-treated groups ($p = 0.001$).

chemia. Although transfection efficiency with plasmids is lower than with adenoviral vectors the level of protein production, at least in the case of VEGF₁₆₅, appears sufficient to obtain meaningful bioactivity as demonstrated by the results of this and other studies (Isner *et al.*, 1998). We believe this is to a large extent because VEGF₁₆₅ contains an NH₂-terminal signal sequence that allows the protein to be secreted from the cell, thus enhancing its potential bioactivity. In addition, VEGF is endothelial cell specific, with its primary receptor (Kdr) being located exclusively on endothelial cells, thus allowing essentially all the VEGF activity to be focused on the cellular element most involved in angiogenesis.

The effect of phVEGF on plasma VEGF protein production was evident within 2 days and peaked at about 4–5 days after gene transfer. However, individual values were highly variable. At the tissue level, however, the greatest concentration of VEGF was consistently seen closest to the injection site. Remarkably, protein was detectable after transfection in nonischemic pig hearts as long as 8 weeks after treatment. This is consistent with intramyocardial injection studies performed in rodents (Buttrick *et al.*, 1992; Gal *et al.*, 1993). VEGF was also detectable in the plasma and myocardium of ischemic pigs in the control group.

Comparison of myocardial blood flow to the ischemic circumflex territory demonstrated similar findings at baseline in both the control and VEGF-treated animals. While vasodilation with adenosine did not augment flow to the ischemic territory in control animals a significant improvement was seen in the VEGF group. Interestingly, the magnitude of this augmentation of collateral flow to the territory subtended by the occluded circumflex coronary artery was in fact superior to that achieved in previous experiments from our laboratory in which VEGF₁₆₅ protein was injected into the left coronary artery of the identical animal model (Hariawala *et al.*, 1996).

Alternative techniques for myocardial gene delivery include intracoronary administration and percutaneous catheter-based myocardial injection. While both of these approaches would have the advantage of avoiding the need for a thoracotomy and general anesthesia, intracoronary injection has been shown to

be far less effective in delivering the gene to the myocardium (Lee *et al.*, 1999) and a catheter-based intramyocardial delivery system is still under development.

In conclusion, this study demonstrates that intramyocardial injection of phVEGF165 via a minimally invasive thoracotomy is feasible. It results in sufficient VEGF production to significantly enhance myocardial perfusion in ischemic pig hearts. These data suggest that future clinical trials should utilize direct intramyocardial gene transfer via a minithoracotomy to evaluate the potential benefits of gene therapy with VEGF in patients with advanced coronary disease.

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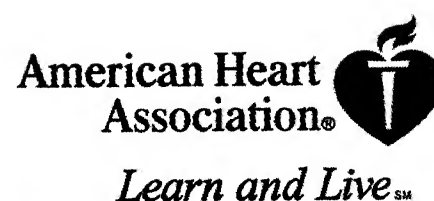
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EXHIBIT L

Circulation

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Expression of recombinant genes in myocardium in vivo after direct injection of DNA

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Brief Rapid Communication

Expression of Recombinant Genes in Myocardium In Vivo After Direct Injection of DNA

Hua Lin, MD, Michael S. Parmacek, MD, Gerald Morle, BS,
Steven Bolling, MD, and Jeffrey M. Leiden, MD, PhD

The ability to program recombinant gene expression in cardiac myocytes in vivo holds promise for the treatment of many inherited and acquired cardiovascular diseases. In this report, we demonstrate that a recombinant β -galactosidase gene under the control of the Rous sarcoma virus promoter can be introduced into and expressed in adult rat cardiac myocytes in vivo by the injection of purified plasmid DNA directly into the left ventricular wall. Cardiac myocytes expressing recombinant β -galactosidase were detected histochemically in rat hearts for at least 4 weeks after injection of the β -galactosidase gene. These results demonstrate the potential of this method of somatic gene therapy for the treatment of cardiovascular disease. (*Circulation* 1990;82:2217-2221)

Somatic gene therapy, the expression of recombinant genes in non-germ-line tissues of the adult organism, holds great promise for the treatment of many inherited and acquired human diseases (reviewed in Reference 1). The biological requirements for this type of gene therapy include the ability to introduce recombinant genes efficiently into the appropriate cells and tissues and to program the high-level and, in many cases, stable expression of these recombinant genes in vivo. In addition, it is necessary that the process of gene therapy itself not be harmful to the recipient organism, in particular, that the techniques used to introduce the recombinant genes do not result in persistent infection of the host or in deleterious mutations of the recipient cells. Two general approaches have proven useful in animal models of somatic gene therapy. In the first, recombinant genes have been introduced into cultured cells in vitro, and cells expressing the recombinant gene product have then been transplanted into the appropriate tissue of a recipient animal.²⁻⁴ In the second, recombinant genes have been introduced directly into somatic cells in vivo.⁵

The ability to program recombinant gene expression in adult myocardium in vivo requires both an expression vector with high-level activity in cardiac myocytes and a method for introducing such a vector into myocardial cells in the adult animal. A previous study demonstrated that murine skeletal myocytes display a rather unique ability to take up and express DNA after direct injection in vivo.⁶ In the studies described in this report, we show that an expression vector using the Rous sarcoma virus (RSV) long terminal repeat (LTR) programs high-level recombinant gene expression in rat cardiac myocytes in vitro and demonstrate that recombinant genes cloned into this vector can be introduced into and expressed in adult rat cardiac myocytes for at least 4 weeks after direct injection of plasmid DNA into the left ventricular wall.

Methods

Cell Culture and Transient Transfections

Neonatal rat cardiac myocytes were isolated from 1-2-day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) by collagenase digestion as previously described.⁷ This method results in the isolation of more than 90% cardiac myocytes.⁷ Twenty-four hours after isolation, 1×10^6 freshly isolated myocytes in a 60-mm collagen-coated dish (Collaborative Research Inc., Waltham, Mass.) were transfected with 15 μ g of cesium chloride gradient-purified chloramphenicol acetyl transferase (CAT) reporter plasmid DNA plus 5 μ g of pMSV β gal reference plasmid DNA as follows: 20 μ g of plasmid DNA was resuspended in 1.5 ml of Opti-MEM (GIBCO, Grand Island, N.Y.)

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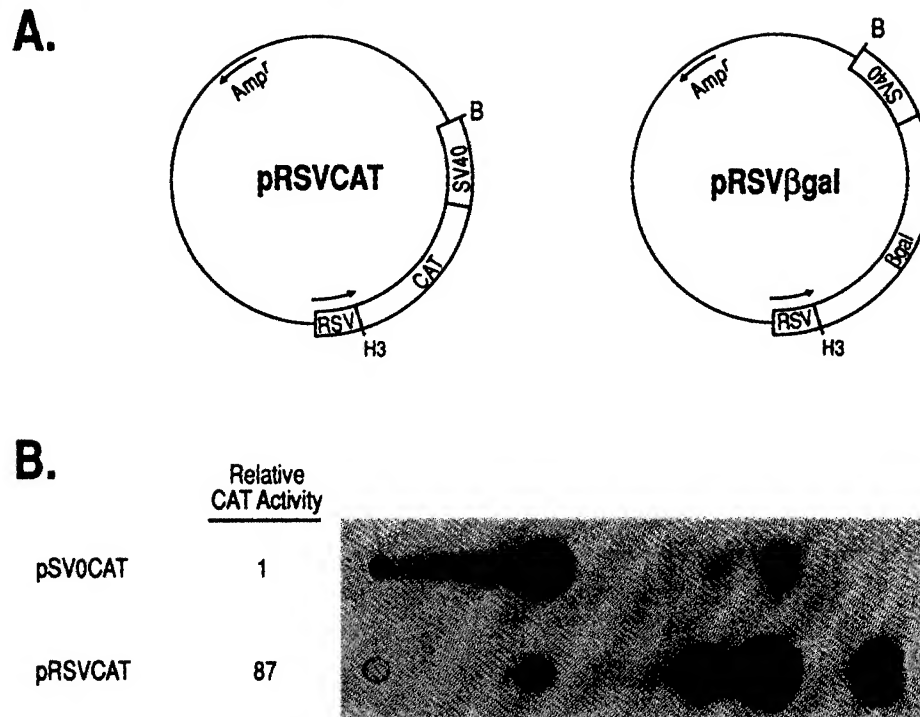


FIGURE 1. Transcriptional activity of the Rous sarcoma virus (RSV) long terminal repeat (LTR) in rat neonatal cardiocytes *in vitro*. Panel A: A schematic representation of the pRSVCAT and pRSV β gal plasmids. β gal, β -galactosidase gene; CAT, chloramphenicol acetyl transferase gene. HindIII (H3) and BamHI (B) restriction endonuclease sites are shown. Panel B: Transcriptional activity of the RSV LTR in rat neonatal cardiocytes *in vitro*. Rat neonatal cardiocytes were transfected with 15 μ g of the promoterless pSVOCAT control plasmid or the pRSVCAT plasmid (see panel A) and cell extracts prepared 48 hours after transfection were normalized for protein content and assayed for CAT activity as previously described.⁹ To control for differences in transfection efficiencies, all transfections also contained 5 μ g of the pMSV β gal reference plasmid. Data are shown as CAT activity relative to that produced by the pSVOCAT plasmid (which produced 1.7% acetylation) after correction for differences in transfection efficiency.

and added to 1.5 ml of Opti-MEM containing 50 μ l of lipofectin reagent (BRL, Gaithersburg, Md.). The resulting mixture was added to one 60-mm plate of cardiac myocytes. After 5 hours at 37° C in 5% CO₂, 3 ml of Medium 199 plus 5% fetal bovine serum (FCS) (GIBCO) was added to the cells, and the mixture was incubated at 37° C for 48 hours. Cell extracts were prepared and normalized for protein content using a commercially available kit (Biorad, Richmond, Calif.). CAT and β -galactosidase assays were performed as previously described.⁹

Plasmids

The promoterless pSVOCAT plasmid¹⁰ and the pRSVCAT¹¹ plasmid in which transcription of the bacterial CAT gene is under the control of the RSV promoter have been described previously. The pRSV β gal plasmid was constructed by cloning the 4.0-kb β -galactosidase gene from pMSV β gal¹² into HindIII/BamHI-digested pRSVCAT (see Figure 1A).

Injection of Recombinant DNA *In Vivo*

Six- to 11-week-old 250-g Sprague-Dawley rats were housed and cared for according to National Institutes of Health guidelines in the ULAM facility of the University of Michigan Medical Center. Rats were anesthetized with 20 mg/kg pentobarbital i.p. and 60

mg/kg ketamine i.m., intubated, and ventilated with a Harvard (Harvard Apparatus, South Natick, Mass.) respirator. A left lateral thoracotomy was performed to expose the beating heart, and 100 μ g of plasmid DNA in 100 μ l of phosphate-buffered saline (PBS) containing 5% sucrose (PBS/sucrose) was injected into the apical portion of the beating left ventricle using a 30-g needle. Control animals were injected with 100 μ l of PBS/sucrose alone. The animals were killed 3–5 or 21–30 days after injection by pentobarbital euthanasia; hearts were removed via a median sternotomy, rinsed in ice-cold PBS, and processed for β -galactosidase activity.

Histochemical Analysis

Three-millimeter cross sections of the left ventricle were fixed for 5 minutes at room temperature with 1.25% glutaraldehyde in PBS, washed three times at room temperature in PBS, and stained for β -galactosidase activity with X-gal (Biorad) for 4–16 hours as described by Nabel et al.² The 3-mm sections were embedded with glycomethacrylate, and 4–7- μ m sections were cut and counterstained with hematoxylin and eosin as described previously.² Photomicroscopy was performed using Kodak Ektachrome 200 film and Leitz Laborlux D and Wild M8 microscopes.

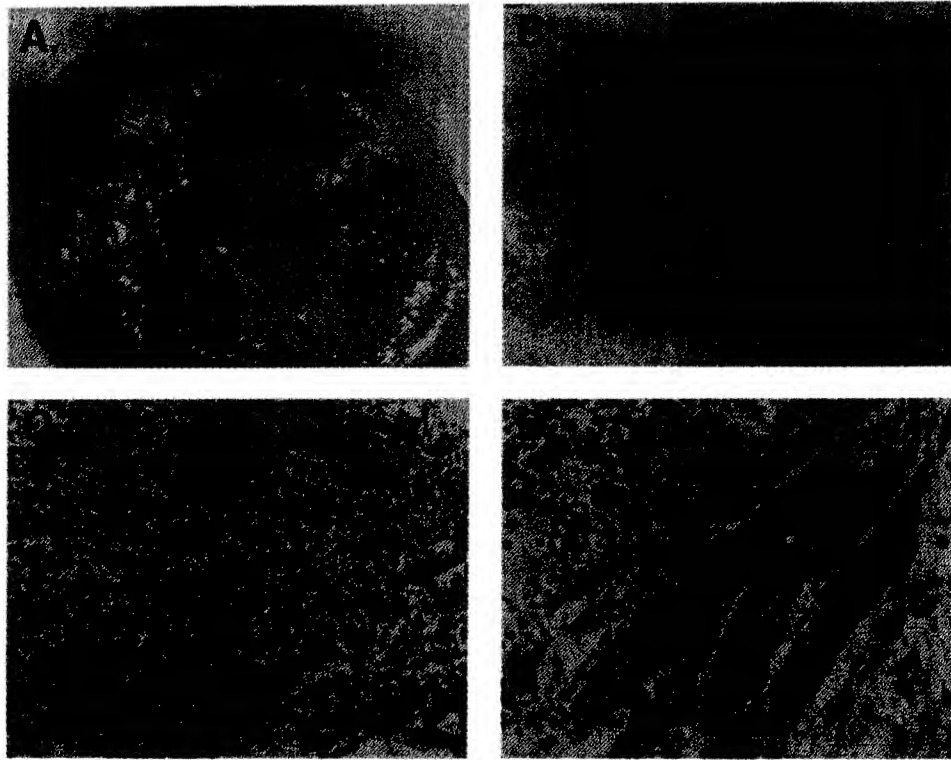


FIGURE 2. Expression of a recombinant β -galactosidase gene in cardiac myocytes *in vivo* after direct injection of pRSV β gal DNA into the left ventricular wall. One hundred micrograms of pRSV β gal DNA was injected into the beating apical wall of the left ventricle of Sprague-Dawley rats using a 30-g needle as described in "Methods." Hearts were harvested 3–5 days or 3–4 weeks after injection and stained for β -galactosidase activity. Panel A: 10 \times view of a 3-mm section of a heart 3 days after pRSV β gal injection. Panel B: 18 \times view of a 3-mm section from a heart 27 days after pRSV β gal injection. Panels C and D: 125 \times and 250 \times views, respectively, of 4- μ m sections from a heart 3 days after pRSV β gal injection. β -Galactosidase activity (dark-blue staining) is seen only within cardiac myocytes that can be identified by their myofibrillar architecture.

Results

RSV LTR Promotes High-Level Gene Expression in Rat Neonatal Cardiocytes In Vitro

Although the RSV LTR displays high-level transcriptional promoter activity in a wide variety of immortalized cell types,¹¹ previous transgenic studies have suggested that this promoter is preferentially active in skeletal and cardiac myocytes *in vivo*.^{13,14} To test directly the transcriptional activity of the RSV LTR in rodent cardiac myocytes, the pRSVCAT vector¹¹ in which expression of the bacterial CAT gene is under the control of the RSV LTR was transfected into primary neonatal rat cardiac myocytes using lipofectin. Two days after transfection, the cultures were harvested and assayed for CAT activity as previously described.⁹ All transfections also contained 5 μ g of the pMSV β gal plasmid¹² to correct for differences in transfection efficiencies. As shown in Figure 1, the RSV LTR was able to increase transcription of the CAT gene 87-fold compared with the promoterless pSVOCAT control plasmid. The pRSVCAT-transfected cardiac myocyte extracts produced 95% acetylation in a standard thin-layer chromatography assay.⁹ By

comparison, identically prepared extracts of 3T3 or HeLa cells transfected with this same vector produced 22% and 35% acetylation, respectively (data not shown). Because the activities of cotransfected pMSV β gal reference plasmids were almost identical in all three transfections, these results demonstrated that the RSV LTR programs high-level transcription in primary cardiac myocytes *in vitro*.

The ability to unambiguously identify the cell types that are expressing recombinant gene products is an important requirement of all animal models of gene therapy. Because the bacterial β -galactosidase reporter gene (but not the bacterial CAT gene) allows direct histological visualization of recombinant gene expression, we constructed a pRSV β gal vector in which bacterial β -galactosidase gene expression is regulated by the RSV LTR promoter for further studies of recombinant gene expression *in vivo* (Figure 1B).

Expression of β -Galactosidase Gene in Rat Cardiac Myocytes After Injection of pRSV β gal DNA Into the Left Ventricular Wall In Vivo

In an attempt to program recombinant β -galactosidase gene expression in rat cardiac myocytes in

vivo, we took advantage of a previously described technique for producing recombinant gene expression in murine skeletal myocytes *in vivo*.⁶ Briefly, 100 μ g of pRSV β gal DNA was resuspended in 100 μ l of PBS containing 5% sucrose (PBS/sucrose) and injected via a 30-g needle directly into the beating left ventricular wall of 6–11-week-old Sprague-Dawley rat hearts. Control rats received injections of 100 μ l of PBS/sucrose without DNA. Rats were killed either 3–5 days or 3–4 weeks after injection, and hearts were fixed and stained for β -galactosidase activity. β -Galactosidase activity as manifested by dark-blue staining was readily apparent to the naked eye in sections of three of four of the pRSV β gal-injected hearts at 3–5 days and four of five of the pRSV β gal-injected hearts at 3–4 weeks after DNA injections (Figures 2A and 2B). This staining, which was focal and patchy, occurred only in a single area of each heart injected with pRSV β gal DNA and was not seen in five control hearts injected with PBS/saline alone (data not shown). Failure to observe staining in two of nine of the pRSV β gal-injected hearts may have been due to the lack of DNA uptake or expression in these hearts or, more likely, to technical difficulties in successfully centering and anchoring the needle in the relatively thin beating left ventricular wall during the injection process.

Because the normal ventricular wall contains both myocytes and fibroblasts and because the injection of DNA might be expected to cause a localized inflammatory response, it was important to determine which cell types were expressing the recombinant β -galactosidase gene. Histochemical analysis of sections from hearts injected with the pRSV β gal DNA clearly demonstrated β -galactosidase activity within cardiac myocytes that were easily identified by their myofibrillar architecture (Figures 2C and 2D). Between one and 10 positively staining myocytes were seen per high-power field, and these were often noncontiguous, suggesting that the uptake of DNA and/or its expression is a relatively low-frequency event. Because it was difficult to accurately identify the extent of DNA injection and because the positively staining areas were quite focal and patchy, it was impossible to accurately quantitate either the percentage or the total number of cells expressing recombinant β -galactosidase activity in a given heart. However, it is clear that only a small fraction of cardiac myocytes expressed the recombinant protein. In addition, it is worth noting that sections from the 3–5-day postinjection hearts often showed evidence of an acute inflammatory response along the track of the needle (Figure 2C) and that in several cases fibrosis along the needle track was observed in sections from the 3–4-week postinjection hearts (data not shown).

Discussion

The studies presented have demonstrated that it is possible to program recombinant gene expression in cardiac myocytes after direct injection of DNA into the left ventricular wall. Functional recombinant protein expression in myocytes was demonstrated

directly using an enzymatic assay for β -galactosidase. Recombinant gene expression was observed in myocytes from seven of nine of the injected hearts at both 3–5 days and 3–4 weeks after injection. Expression was patchy and was observed only in direct contiguity with the site of injection. These findings have several implications regarding both the use of this method for somatic gene therapy in the heart and the biology of recombinant DNA uptake and expression in muscle cells.

A previous study suggested that murine skeletal muscle cells possess a unique ability to take up and express injected recombinant DNA.⁶ Our results have extended this observation to cardiac muscle cells in a second rodent species. It has previously been thought that successful DNA transfection and expression may require recipient cell division and, more specifically, breakdown of the recipient cell nuclear membrane to allow DNA entry. Because skeletal myocytes have a limited potential for mitosis,¹⁵ it remained possible that the previously reported successful transfection of skeletal myocytes was dependent on their mitotic potential. In contrast to skeletal myocytes, adult cardiac muscle cells are unable to divide.¹⁶ Thus, our results demonstrate that mitosis is not necessary for successful transfection of cells with DNA. The mechanisms that allow preferential uptake of DNA into cardiac and skeletal myocytes remain unclear. However, our data suggest that they must be dependent on structural or functional properties that are shared by skeletal and cardiac muscle. Current hypotheses include the possibility of specialized muscle cell transport systems or the unique ability to physically disrupt the cell membranes of muscle cells in a reversible fashion during the recombinant DNA injections.

The technique of somatic gene therapy using direct DNA injection into myocardium, as described in this report, has several advantages compared with other previously described methods of gene therapy. First, infectious viral vectors are not required, eliminating the possibility of persistent infection of the host. Second, a previous study⁶ has suggested that recombinant DNA taken up and expressed in skeletal myocytes persists as an episome and therefore does not have the same potential for host cell mutagenesis as do retroviral vectors that integrate into the host chromosome. Finally, this method does not require the growth of recipient cells *in vitro*, a requirement that would render transfection of nondividing cardiac myocytes particularly difficult.

Direct injection of recombinant DNA into the myocardium holds promise for the treatment of many acquired and inherited cardiovascular diseases. We are particularly interested in the possibility of stimulating collateral circulation in areas of chronic myocardial ischemia by expressing recombinant angiogenesis factors locally in the ventricular wall. Although the method described in this report is a first step toward such gene therapy approaches, many questions and problems remain to be addressed

before this type of gene therapy can become a reality. First, it must be demonstrated that human myocytes, like their rodent counterparts, are able to take up and express recombinant DNA. The longevity of recombinant gene expression must be more fully examined, and the possibility that some of the recombinant DNA is integrated into the host genome with the concomitant potential for mutagenesis must be ruled out. Modifications of the current transfection protocol must be developed to increase the frequency of recombinant gene expression in cardiac myocytes. Of equal importance, the inflammatory response to the injected DNA must be controlled to prevent the formation of arrhythmogenic foci. Finally, it will of interest to determine whether high-level recombinant gene expression can be programmed *in vivo* by the injection of expression vectors containing cardiac-specific transcriptional regulatory elements. Ongoing studies in our laboratory are designed to address these problems. Nevertheless, the initial studies described in this report suggest that somatic gene therapy in the heart may eventually become a useful therapeutic modality.

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KEY WORDS • gene therapy • DNA

EXHIBIT M

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Behavior of Genes Directly Injected Into the Rat Heart In Vivo

Peter M. Buttrick, Alyson Kass, Richard N. Kitsis,
Matthew L. Kaplan, and Leslie A. Leinwand

Gene transfer can be achieved in the adult rat heart in vivo by direct injection of plasmid DNA. In this report we define the spatial and temporal limits of reporter gene expression after a single intracardiac injection. pRSVCAT (100 μ g), in which the Rous sarcoma virus long terminal repeat is fused to the chloramphenicol acetyltransferase reporter gene, and p α MHCluc (100 μ g), in which the α -cardiac myosin heavy chain promoter is fused to the firefly luciferase gene, were injected into hearts, and reporter gene activities were assayed at various times. Both chloramphenicol acetyltransferase and luciferase were detectable in 100% of the rats from 1 to 7 days, in 60% of the rats from 17 to 23 days, and in 30% of the rats from 38 to 60 days after injection. Reporter gene activity was largely limited to a 1–2-mm region of the ventricle surrounding the injection site. Closed circular DNA was far more effective than linear DNA in transfecting cells in vivo. The relative strengths of three different promoters, Rous sarcoma virus long terminal repeat, α -myosin heavy chain, and α_1 -antitrypsin, all fused to the luciferase reporter gene were determined. The constitutive viral promoter was \sim 20-fold more active than the cardiac-specific cellular promoter, and the liver-specific cellular promoter was not active at all in the cardiac environment. Thus, direct injection of genes into the heart offers a simple and powerful tool with which to assess the behavior of genes in vivo. However, the potential of the technique to effect a phenotypic change in the heart is currently limited by the temporal and geographic extent of transfection. (*Circulation Research* 1992;70:193–198)

It has previously been demonstrated that reporter genes linked to viral and cellular promoters can be expressed after injection of plasmid DNA into the hearts of adult rats in vivo.^{1–3} Although this technique suggests a simple approach to somatic gene therapy, its utility is dependent on several parameters. One of these is the stability of the transfected gene. Acsadi et al² recently reported that reporter genes coupled to viral promoters could not be detected in adult cardiac muscle 3 weeks after injection, suggesting that cardiac gene transfer may not be stable. In contrast, Lin et al³ were able to detect reporter gene activity up to 3–4 months after cardiac injection in a small number of animals. Whether the introduced gene is episomal may affect (either positively or negatively) the stability of muscle gene transfer, and the postmitotic state of adult

muscle cells may prevent introduced genes from integrating into chromosomes. In fact, Wolff et al⁴ have suggested that, both in heart and skeletal muscle, directly transfected genes are episomal.

A second consideration for cardiac gene therapy is the spatial extent of transfection after a single injection. Although this has not been investigated in great depth, the precise number of cells transfected after a single injection appears to be small, based on the extent of β -galactosidase staining in tissue sections from hearts injected with this reporter gene.^{2,3} Acsadi et al² have shown that no reporter gene activity can be detected in atria after intraventricular injections.

In the present study, we sought to further define some of the parameters of cardiac gene transfer. Specifically, we determined the time course and geographic extent of gene expression after a single intracardiac injection. In addition, we asked whether the state of the injected DNA, either linear or circular, influenced its expression. Finally, we determined the relative strengths of a constitutive viral promoter, a cardiac-specific cellular promoter, and a liver-specific promoter. Transcription from the viral long terminal repeat (LTR) was \sim 20-fold higher than that derived from the cardiac promoter, but no transcription resulted from the liver-specific promoter.

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Materials and Methods

The plasmids used in this study have been previously described: α MHCluc contains the firefly luciferase coding region coupled to the rat cardiac α -myosin heavy chain 5' flanking sequence, base pairs -613 to +32.⁵ pRSVCAT contains the chloramphenicol acetyltransferase (CAT) coding sequence spliced to the LTR of the Rous sarcoma virus (RSV), and pRSVluc contains the firefly luciferase coding sequence coupled to the RSV-LTR.⁶ α ATluc contains the luciferase coding region coupled to the 5' flanking sequence, base pairs -522 to +20, of the α ₁-antitrypsin gene. This construct has significant promoter activity when transfected into cultured hepatocytes.⁷

Adult female Wistar rats were anesthetized with an intraperitoneal injection of chloral hydrate (0.7 ml/100 g of a 4% solution). Cardiac injections were made directly into the apex of the left ventricle after exteriorization of the heart through a left lateral thoracotomy, after which the heart was replaced in the chest, the rats were briefly hyperventilated, and the incision was closed. Fifty microliters of a DNA solution containing 2 μ g/ μ l of each plasmid in 20% sucrose and 2% Evans blue were injected through a 27-gauge needle.

Rats were killed 5 days after injection except when indicated. The atria and great vessels were trimmed, and the hearts were washed in iced saline. Except when indicated, the apical two thirds of the heart was homogenized in 1 ml homogenization buffer without Triton X-100, as described,⁸ with a Tissumizer (Tekmar, Cincinnati, Ohio). Homogenates were then centrifuged at 6,000g for 10 minutes at 4°C.

CAT assays were done on 5% of the supernatant of each homogenate by standard techniques⁹ with the following modifications. Samples were first heated to 65°C for 10 minutes and then clarified by centrifugation at 10,000g for 5 minutes. Reaction mixtures were incubated for 2 hours at 37°C, and percent CAT conversion was determined by excising the appropriate areas of the thin-layer chromatography plate and measuring the associated radioactivity directly in Econofluor. Luciferase was also measured in 5% of the homogenate as previously described.⁸ Light production after the addition of D-luciferin to the assay mix was measured for 20 seconds and integrated over time in a Monolight luminometer (model 2010, Analytical Luminescence Laboratory, San Diego, Calif.).

Results

To determine the time course of gene expression after direct intracardiac injection, the rats were injected with 100 μ g each of pRSVCAT and α MHCluc and then killed at various time points. Data for individual rats are shown in Figure 1. No reporter gene activity was detected 2 hours after injection; however, at 1 day all injected hearts showed both CAT and luciferase activities. The levels of expression continued to rise and reached maximal

levels 7–10 days after injection. All injected hearts showed significant reporter gene activities up to this point. At subsequent time points, some of the hearts showed no detectable reporter gene activities: four of 10 hearts from 17 to 23 days and seven of 10 hearts from 38 to 60 days. Southern analysis (not shown) of *Hind*III digests of cellular DNA suggested that the exogenous genes were episomal.

We determined the ratio of luciferase activity to CAT activity in hearts coinjected with pRSVCAT and α MHCluc as a function of time in order to compare the behavior of these two reporter genes. In general, this ratio was constant over the first 2 weeks after injection: 424 ± 67 in samples from 1 to 2 days versus 484 ± 104 in samples from 7 to 23 days. In the hearts expressing the reporter genes at later time points (from 38 to 60 days), the ratio of luciferase to CAT activity fell significantly to 77 ± 20 .

To determine the spatial extent of expression of injected genes in cardiac muscle, we injected the apical portions of individual hearts with a single 50- μ l bolus of pRSVluc, a plasmid containing the luciferase reporter gene coupled to the constitutive RSV-LTR. Five days after injection, the rats were killed, and the hearts were cut into 1-mm sections from apex to base. These sections were then assayed individually for the presence of luciferase activity. Data from two hearts are shown in Figure 2 and indicate that 90% of the expression of the luciferase gene was restricted to two 1-mm sections surrounding the injection site, although very low levels of activity were seen in two other 1-mm sections, so that some minimal activity was detectable over 40% of the long axis of the ventricle. No reporter gene activity was seen in the atria.

To assess whether the state of the injected DNA influenced its expression, we compared luciferase activity after injection with linear versus closed circular DNA. α MHCluc was digested to completion with *Xmn* I, which does not disrupt the promoter and coding regions of the plasmid, and 100 μ g of this linear molecule was coinjected with 100 μ g closed circular pRSVCAT (as an internal control) in four rats and compared with the uncut circular plasmid, which was also coinjected with pRSVCAT in a similar number of rats. Figure 3 shows the percent CAT conversions, luciferase activities, and luciferase/CAT ratios in these rats. Luciferase/CAT ratios (Figure 3C) were 50–100-fold greater in those rats injected with circular DNA than in rats injected with linear DNA, demonstrating that the linear molecule was poorly expressed relative to the closed circular plasmid. CAT activities tended to be lower in the rats concomitantly injected with the linear molecule (Figure 3B), suggesting that this interfered with pRSVCAT uptake and/or transcription.

Finally, we determined the relative strengths of three promoters linked to the luciferase reporter gene in the heart. The promoters were derived from the RSV-LTR, the rat α -cardiac myosin heavy chain gene, and the liver-specific mouse α ₁-antitrypsin gene. In this experiment, 100 μ g each pRSVluc, α MHCluc, or

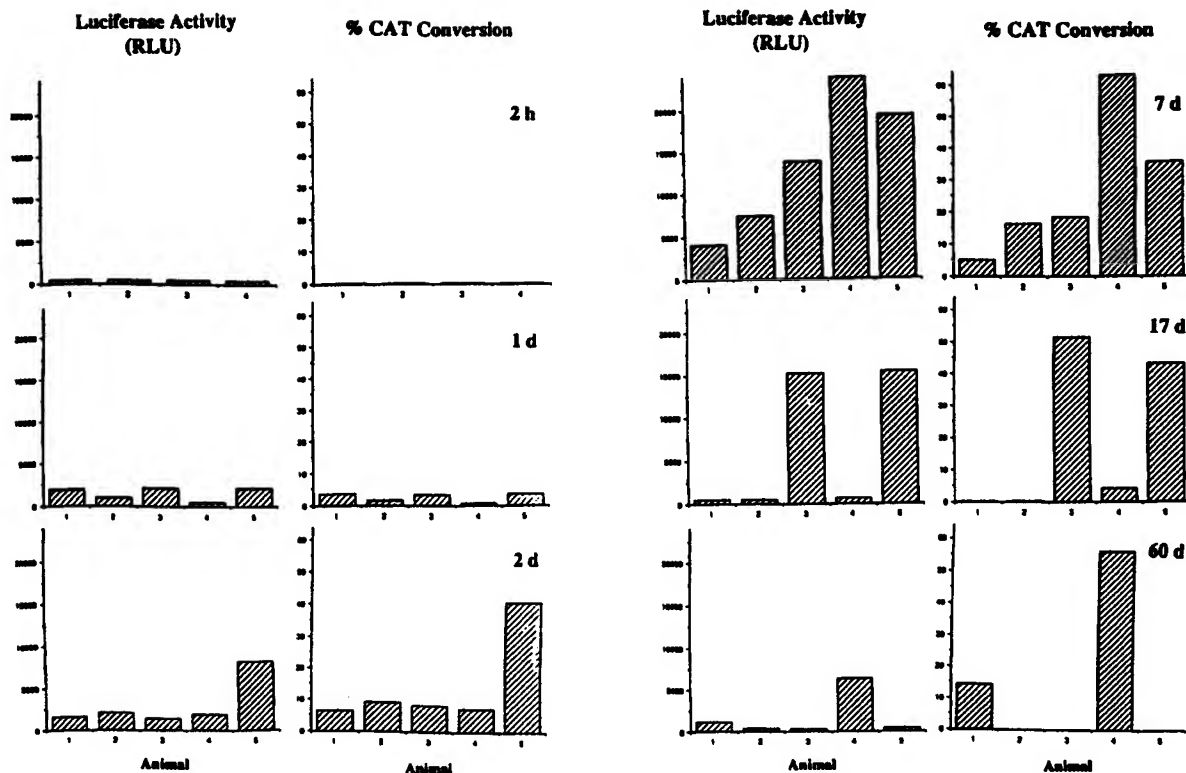


FIGURE 1. Bar graphs showing expression of coinjected genes at six time points (from 2 hours to 60 days) after direct cardiac injection of plasmids pRSVCAT and p α MHCluc. Luciferase activity (raw luciferase units [RLU]) and percent chloramphenicol acetyltransferase (CAT) conversion are shown for individual heart homogenates at each time point.

p α 1ATluc was coinjected into individual hearts along with 15 μ g pRSVCAT. The rats were killed 5 days after injection, and reporter gene activities were assayed. Luciferase activity was normalized to CAT activity. The data are shown in Table 1 and demonstrate that the viral promoter (RSV) was ~20-fold more potent than the cardiac-specific cellular promoter (α -myosin heavy chain) and, in addition, that the liver-specific promoter (α ₁-antitrypsin) was not active in the cardiac environment.

Discussion

This study describes the time course and spatial extent of gene expression in the rat ventricle after direct intracardiac injection of plasmid DNA. One major finding is that reporter gene activities can be reliably detected as early as 1 day and are stable for

the first 2 weeks after injection. The proportion of rats expressing injected genes from 17 to 23 days is reduced to 60% and from 38 to 60 days to 30%, but in those rats expressing genes, the level of expression is quite high and appears to depend on the continued presence of the episomal plasmid.

The time course of expression of exogenous genes shown in the present study differs from that previously reported in the heart as well as in other tissues. Acsadi et al² reported no significant activity in nine of 10 normal adult Sprague-Dawley rats 25 days after cardiac injection, although a higher percentage of rats, similar to that seen in our study with Wistar rats, did express reporter genes at this time point if the rats were concomitantly treated with cyclosporin. This suggests that strain differences might contribute to the observed results. Other workers have shown

TABLE 1. Relative Promoter Strengths in Cardiac Muscle In Vivo

Promoter	n	Luciferase activity (RLU)		CAT conversion (%)		Luciferase/CAT	
		Mean \pm SEM	Range	Mean \pm SEM	Range	Mean \pm SEM	Range
RSV	5	287,659 \pm 82,058	42,546–464,327	3.3 \pm 1.0	0.4–5.6	96,841 \pm 17,629	56,034–162,124
α -MHC	6	73,668 \pm 13,414*	22,882–114,699	15.0 \pm 2.6*	4.0–21.9	5,014 \pm 370*	3,651–5,752
α ₁ -AT	6	162 \pm 38*†	108–349	27.2 \pm 4.3*†	15.2–42.8	8 \pm 3*†	3–21

n, Number of rats; RLU, raw luciferase units; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; α -MHC, α -myosin heavy chain; α ₁-AT, α ₁-antitrypsin. The three promoters (100 μ g each) coupled to the luciferase gene were coinjected with 15 μ g pRSVCAT into rat hearts in vivo. Reporter gene activities were measured 5 days later.

* p <0.05 vs. corresponding value for RSV; † p <0.05 vs. corresponding value for α -MHC.

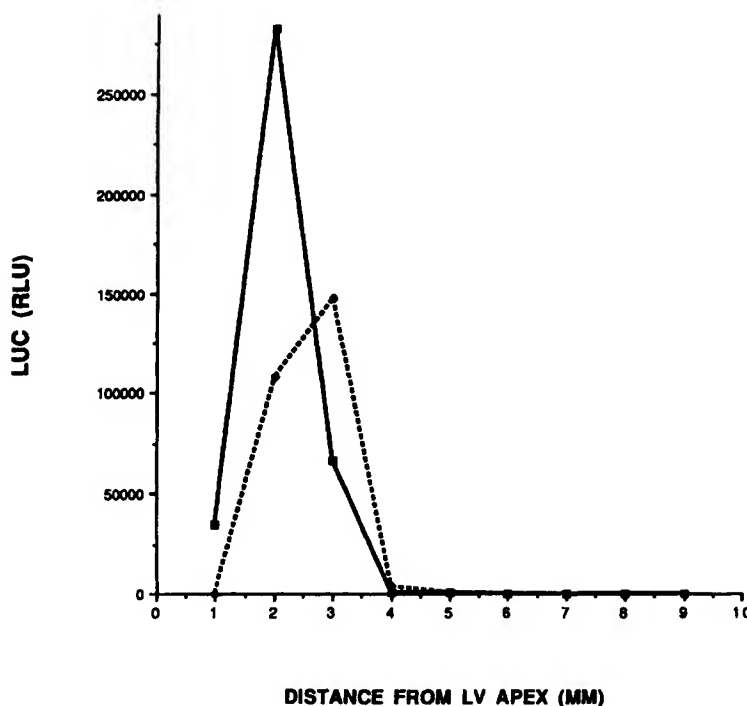


FIGURE 2. Graph showing the geographic extent of gene expression after direct cardiac injection of the plasmid pRSVluc in two individual hearts. The entire heart was sectioned 5 days after the injection of DNA. The distance from the left ventricular (LV) apex of the heart (mm) is indicated on the abscissa, and the luciferase (LUC) activity (raw luciferase units [RLU]) in individual heart slices is indicated on the ordinate.

that transfection of exogenous genes into both skeletal and vascular muscle results in more stable levels of gene expression. Mouse gastrocnemius muscles express exogenous genes 60 days after injection at levels that are similar to those seen at 7–15 days,⁴ and pig iliac arteries infected with a modified retroviral vector bearing the β -galactosidase gene show some activity 3–6 months after treatment.¹⁰ This latter result may reflect plasmid integration since some cellular hyperplasia was observed after endothelial injury.

The current study also demonstrates that, after a single injection, expression of reporter genes is localized to a relatively small area of myocardium. The fact that the myocyte is the transfected cell derives from other histological studies that have localized reporter gene expression to this cell type^{2,3} and from our previous studies showing tissue-restricted expression of reporter genes driven by a cardiac myocyte-specific cellular promoter.¹ Although the spatial extent of gene transfer after a single injection is limited, it may be possible to achieve gene transfer into a larger area of the myocardium using multiple injections or agents that may improve DNA uptake and delivery.

The mechanism whereby DNA is taken up by striated muscle is unknown. The demonstration that linear DNA does not result in efficient gene transfer *in vivo* may not be surprising, given its inefficient uptake by cultured cells in transfection experiments. However, these two gene transfer systems may be distinct, and given the results of Wolff et al,⁴ who reported that linear RNA is effective in skeletal muscle gene transfer, the finding is an important one.

An additional piece of information provided in the present study is the first quantitative assessment of the relative strengths of promoters *in vivo*. Our data indicate that the constitutive RSV-LTR is ~20-fold more active than a cardiac-specific cellular promoter. The absence of any discernible promoter activity after injection of a liver-specific cellular promoter in the heart confirms our previous demonstration¹ that directly injected cellular genes behave in a tissue-restricted fashion and also establishes that the heart is not a promiscuous environment for gene expression via this technique. An additional finding from this experiment is that competition for transcription factors likely occurs when two strong promoters are coinjected into cardiac muscle. This is striking when contrasting CAT activities in the rats injected with pRSVluc/pRSVCAT (3.3% conversion) and p α MHCluc/pRSVCAT (15.0% conversion) but is also evident when comparing these two groups with the rats injected with p α ATluc/pRSVCAT (27.2% conversion). Similar competition among promoters has been observed in cultured cells (e.g., see Reference 11). These findings are important when considering the utility of this approach for *in vivo* promoter mapping.

There are several potential applications of this technology. One is to alter cardiac phenotype through somatic cell gene therapy and a second is to identify regulatory regions of genes that respond to stimuli that can only be modeled *in vivo*, such as hypertension or pressure overload. On the basis of the current data, the first of these may have limited feasibility. Gene expression after a single injection using the current approach is temporally variable. The spatial restriction of cells expressing injected

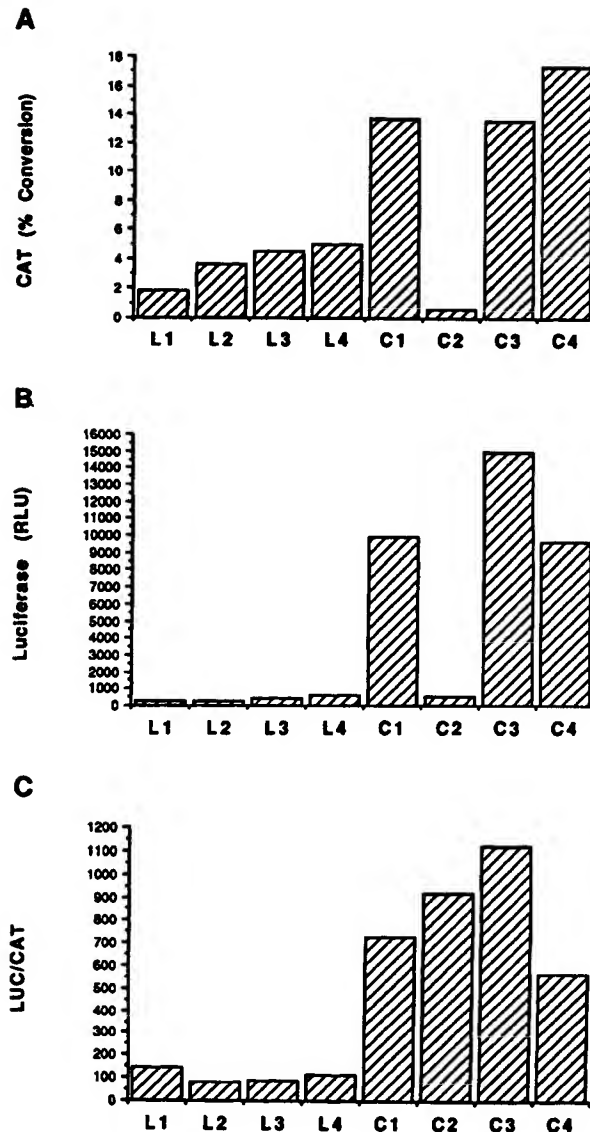


FIGURE 3. Bar graphs comparing gene expression after coinjection with either linear (L) or closed circular (C) plasmids *paMHCluc* and *pRSVCAT* (see text). Data are from four individual rats in each group killed 5 days after injection. Panel A: Chloramphenicol acetyltransferase (CAT) activity. Panel B: Luciferase activity (raw luciferase units [RLU]). Panel C: Luciferase/CAT ratios (LUC/CAT).

genes makes it unlikely that a given gene product will effect a prolonged, global phenotypic change, with the possible exception of transfection with genes such as angiotensin, which may function in an autocrine or paracrine fashion.¹²

The second application, identification of regulatory elements of genes *in vivo*, appears to be possible, and the current data define caveats that ought to be observed for such an experimental approach. First, a constitutively expressed control expression vector must be used as an internal control. The variability in the expression of an individual reporter gene can be

quite significant, which likely reflects injection technique. This is most striking in Figures 3A and 3B, which show significant interanimal variability in CAT and luciferase expression after a single injection. However, when the expression of a cellular promoter (α -cardiac myosin heavy chain) is normalized to that of a constitutive promoter (RSV-LTR) as in Figure 3C, the data are remarkably consistent. The standard error of these four measurements is <15%. Similar experimental variability can be seen in the time course data shown in Figure 1. Second, comparisons of reporter genes should be done during periods of stable gene expression. In the case of the two reporter gene constructs used in the present study, this period is 2–7 days after injection, during which time expression is consistent in all injected hearts and the ratio of CAT/luciferase is constant. At later times, the ratio of the two reporter genes decreases, which may reflect a longer half-life of the CAT protein or preferential transcription of the RSV-LTR-driven reporter gene. The half-life of these reporter genes in mammalian tissue is not known, although the half-life of luciferase expression after injection of RNA expressing the luciferase coding region in skeletal muscle is ~12 hours⁴ and that of CAT in mammalian cells is <2 days.¹³ This suggests that expression of the reporter genes at later time points is the result of ongoing transcription and translation. Finally, potential interactions between plasmids must be considered. For example, competition between strong promoters probably occurs *in vivo*, and in the experiment comparing linear and closed circular DNA, the linear molecule appeared to inhibit either the uptake and/or the expression of the coinjected pRSVCAT.

If these caveats are acknowledged, direct injection of DNA appears to offer a simple and powerful tool with which to assess gene behavior *in vivo*. Future studies, aimed at extending the geographic limits of expression and maintaining the integrity of an injected plasmid, may allow the development of a strategy for somatic cell gene therapy in the heart.

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KEY WORDS • *in vivo* gene transfer • cardiac myosin heavy chain
• firefly luciferase • chloramphenicol acetyltransferase

EXHIBIT N

Hormonal modulation of a gene injected into rat heart *in vivo*

(cardiac gene transfer/transfection/gene regulation/thyroid hormone/myosin heavy chain)

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ABSTRACT We demonstrate gene transfer into rat heart *in vivo* by the direct injection of plasmid DNA. Injection of gene constructs driven by retroviral and cellular promoters resulted in detectable levels of reporter gene activities. The cellular promoter and 5' flanking sequence (positions -613 to +32) were derived from the rat α -myosin heavy chain gene whose expression *in vivo* is restricted to cardiac muscle and is positively regulated by thyroid hormone. After DNA injection, activity of the firefly luciferase gene coupled to the myosin heavy chain promoter and regulatory sequence was detected in heart but not in skeletal muscle and was significantly increased in response to thyroid hormone treatment. Consequently, expression of injected genes can be targeted to specific cell types *in vivo* and can be modulated by the hormonal status of the animal. This approach provides a means of mapping the elements of genes that regulate their responses to complex stimuli that cannot be modeled *in vitro*.

Gene transfer into somatic cells of adult animals has been achieved by several means including the introduction of DNA complexed with carriers and infection with viral vectors (1–5). Reporter genes driven by a retroviral promoter have been expressed after direct injection of plasmid DNA into mouse skeletal muscle (6). This straightforward means of gene transfer suggests an approach to somatic cell gene therapy and offers a way of studying gene regulation *in vivo* that is simpler and less costly than the generation of transgenic animals. For example, the mammalian heart has been shown to respond to various hormonal and hemodynamic stimuli with marked alterations in the expression of specific genes (for a review, see ref. 7). Consequently, the direct injection of DNA into the heart of an intact animal might provide a system with which to study the regulation of cardiac genes in various physiologic and pathologic states that cannot be modeled in tissue culture. Further, some cis-acting regulatory elements have been observed to influence gene expression differently *in vitro* than *in vivo* (8).

To address these issues, we asked whether an injected gene could be expressed in cardiac tissue and, if so, whether a gene coupled to a cellular promoter could be expressed at detectable levels and regulated appropriately. Such a finding would suggest an approach for targeting the expression of injected genes to specific cell types and for modulating their expression. The cellular promoter chosen for this study was derived from the rat α -cardiac myosin heavy chain (α -MHC) gene. *In vivo*, the expression of this gene is restricted to the heart (9) and its activity has been shown both *in vivo* (10) and *in vitro* (11, 12) to be positively regulated by thyroid hormone. By co-injecting rat cardiac and skeletal muscles with reporter genes linked to the α -MHC promoter and to a second viral promoter as an internal control, we demonstrated that the

heart can be transfected *in vivo* with greater efficiency than skeletal muscle. Furthermore, we showed that the directly injected α -MHC promoter is active in heart, but not in skeletal muscle, and that its activity is regulated by the thyroid hormone status of the animal.

MATERIALS AND METHODS

Plasmids. α -MHCluc contains the firefly luciferase coding region coupled to the rat α -MHC 5' flanking sequence, base pairs -613 to +32 (13). The latter, which includes a putative thyroid hormone response element (12), had been subcloned into pTZ19R (United States Biochemical) as an *Xba* I-blunted *Eco*RI fragment. A *Hind*III-*Kpn* I fragment of this plasmid was subcloned into pXP2 (14), a promoterless luciferase vector, to construct α -MHCluc. pRSVCAT, in which the coding sequence of the chloramphenicol acetyltransferase (CAT) gene is spliced to the long terminal repeat of the Rous sarcoma virus (RSV), has been described (15). p0-CAT (pCAT-Basic Vector; Promega) is a promoterless CAT construct.

Animal Models. Adult female Wistar rats were anesthetized with inhaled methoxyfluorane and 0.1 ml/100 g (body weight) of ketamine (50 mg/ml) and xylazine (10 mg/ml) intramuscularly. Animals receiving cardiac injections underwent exteriorization of the heart through a left thoracotomy (16) followed by injection of 50 μ l of a solution containing each plasmid at 2 μ g/ μ l as indicated in 20% (wt/vol) sucrose and 2% (vol/vol) Evans blue into the apex of the left ventricle through a 27-gauge needle. After expression of air from the chest, animals were ventilated briefly on a small-animal respirator and the incision was closed. In other rats, the belly of the adductor magnus muscle was injected under direct visualization.

Two protocols were used in experiments involving thyroid hormone manipulations. (i) Rats were rendered hypothyroid by the administration of propylthiouracil (500 mg/liter in drinking water) (17) for 3 weeks, at which point their hearts were co-injected with 100 μ g of pRSVCAT and 100 μ g of α -MHCluc. The animals were then divided into two groups: one was continued on propylthiouracil and a second received 3,5,3'-triiodothyronine [T₃; 200 μ g/100 g (body weight), intraperitoneally] 2 hr after the cardiac injection and daily for 5 days. Five days after the injection, both groups were sacrificed. A third group consisted of age-matched euthyroid animals that were injected with DNA and sacrificed in parallel. (ii) All animals were euthyroid at the time of intracardiac DNA injection. After this injection, one group received T₃ according to the above schedule but the remain-

Abbreviations: CAT, chloramphenicol acetyltransferase; MHC, myosin heavy chain; T₃, 3,5,3'-triiodothyronine.

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der did not. In this experiment, animals were sacrificed 4 days after the injection.

Tissue Homogenization. At the time of sacrifice, heart and skeletal muscles were resected and washed in ice-cold phosphate-buffered saline. The atria and great vessels were trimmed and the basal third of the ventricles were fast-frozen in liquid nitrogen for subsequent RNA analysis. The apical two-thirds of the ventricles and approximately a 3-cm length of skeletal muscle centered around a suture marking the injection site were weighed, minced into 0.5–1.0 ml of ice-cold homogenization buffer as described (18) except for the omission of Triton X-100, and homogenized with a Tekmar Tissumizer (Tekmar, Cincinnati) for 45 sec. After centrifugation at $6000 \times g$ for 10 min at 4°C , supernatants were transferred to new tubes and their volumes were measured.

CAT Assays. Each homogenate (5–20% of the homogenate, constant within a given experiment) was assayed for CAT activity (19). In some experiments, as noted, specimens were first heated at 65°C for 10 min and then clarified by centrifugation at $10,000 \times g$ for 5 min. Reaction mixtures were incubated for 2 hr at 37°C . Percent chloramphenicol conversion was determined by excising appropriate areas of the thin layer chromatography plate and measuring radioactivity in Econofluor (DuPont/NEN).

Luciferase Assays. Each homogenate (5–20% of the homogenate, constant within a given experiment) was assayed for luciferase activity (18) with the following modifications. Triton X-100 was omitted from the homogenization buffer but added prior to the luciferase reactions. The buffer (18) containing D-luciferin (Analytical Luminescence Laboratory, San Diego) was used at $100 \mu\text{l}$ per assay. Light production was measured for 20 sec and integrated over time by a Monolight model 2010 luminometer (Analytical Luminescence Laboratory).

Northern Blot Analysis. Total RNA ($15 \mu\text{g}$) (20) was size-fractionated on a 0.8% denaturing agarose gel (21) and blotted onto a nylon membrane (Biotrans; ICN). The blot was hybridized sequentially with isoform-specific oligonucleotides complementary to the 3' untranslated regions of α -MHC [nucleotides 5860–5883 (22)] and β -MHC [nucleotides 5846–5869 (23)], according to standard methods (21) at 55°C overnight and then washed in $2 \times \text{SSC}$ ($20 \times \text{SSC} = 3 \text{ M NaCl}/0.3 \text{ M sodium citrate}$)/0.2% SDS for 5 min at 55°C . Autoradiography was performed with XAR film (Kodak) for the exposure times indicated.

Serum Thyroxine Determinations. Thyroid-hormone-manipulated animals and controls underwent determination of serum thyroxine levels several hours prior to intracardiac DNA injections and at the time of sacrifice by radioimmunoassay using a kit (Pantex, Santa Monica, CA) previously validated with rat plasma.

RESULTS

Expression of Genes Directly Injected into Rat Heart. The hearts and adductor magnus muscles of adult female rats were co-injected with two plasmid DNAs, $100 \mu\text{g}$ of pRSV-CAT and $100 \mu\text{g}$ of α -MHCluc. pRSV-CAT directs the synthesis of CAT under the control of the RSV long terminal repeat, which is a constitutively active promoter in many mammalian cell types. α -MHCluc directs the synthesis of firefly luciferase under the control of the rat α -MHC promoter and 5' flanking sequence (positions –613 to +32). The endogenous α -MHC gene is expressed only in cardiac muscle *in vivo* (9). pRSV-CAT was co-injected with α -MHCluc to control for differences in DNA uptake and/or expression both between the two tissues and among individual animals. As negative controls, both heart and skeletal muscles were also co-injected with $100 \mu\text{g}$ of pRSV-CAT and $100 \mu\text{g}$ of pXP2

(14), the latter being a construct containing the luciferase gene with no promoter sequence. Five days after injection, muscle homogenates were assayed for CAT and luciferase activities.

CAT activities greater than 0.25% were detectable in 100% of hearts ($n = 37$) and 73% of skeletal muscles ($n = 30$) (Fig. 1). In contrast, the mean CAT activity resulting from injection of p0-CAT, a promoterless CAT vector, was 0.14%. This result confirms that the constitutive RSV promoter was active in both tissues. However, there was a striking difference in CAT activities between the two tissues. The heart expressed this gene at levels 10–100 times higher per mg of total protein than did skeletal muscle. Although the explanation for this differential expression has not yet been established, it was not caused by a tissue-specific inhibitor as reconstitution experiments using noninjected heart and skeletal muscle lysates and known amounts of exogenous CAT demonstrated similar dose-response curves (data not shown). These results demonstrate that the expression of an injected gene can be easily detected in heart muscle.

Tissue-Restricted Expression of an Injected Gene Coupled to a Cellular Promoter. Since α -MHC gene expression *in vivo* is limited to the heart (9), we hypothesized that a gene linked to the α -MHC promoter (α -MHCluc) would be expressed in heart but not in skeletal muscle after direct DNA injection. No α -MHC-promoter-driven luciferase activity was seen in any of 19 injected skeletal muscles. However, the relatively less-efficient expression of pRSV-CAT injected into skeletal muscle as compared to heart would make it very difficult to detect low levels of α -MHC-promoter-driven luciferase activity in skeletal muscles. To control for this variable, luciferase activities were normalized to CAT activities in each heart and skeletal muscle (Fig. 2). We included in our analysis only those skeletal muscles that had been successfully transfected; i.e., those with CAT activities greater than 0.25% conversion or approximately twice background, resulting in the inclusion of 100% of hearts and 78% of skeletal muscles. The luciferase and CAT activities measured in one experiment are shown in Table 1. The luciferase/CAT ratio, therefore, provides a relative measure of the levels of α -MHC promoter driven luciferase expression in the two tissues if it is assumed that reporter gene mRNA and protein turnover do not differ between the tissues. The activity of the α -MHC promoter was significantly ($P < 0.001$) higher in heart than in

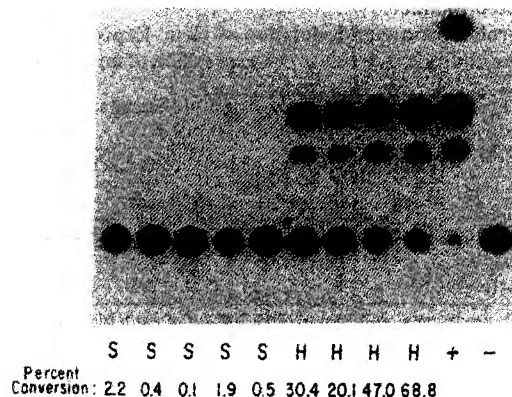


FIG. 1. Heart and skeletal muscles express injected genes. CAT activities in rat skeletal muscles (lanes S) and hearts (lanes H) co-injected with $100 \mu\text{g}$ of pRSV-CAT and $100 \mu\text{g}$ of α -MHCluc as shown. Twenty percent of each organ homogenate was assayed 5 days after DNA injection. The above autoradiogram resulted from a 12-hr exposure. Percent chloramphenicol conversion is indicated below each lane. Lanes + and – correspond to positive and negative controls consisting of homogenization buffer with or without partially purified *Escherichia coli* CAT.

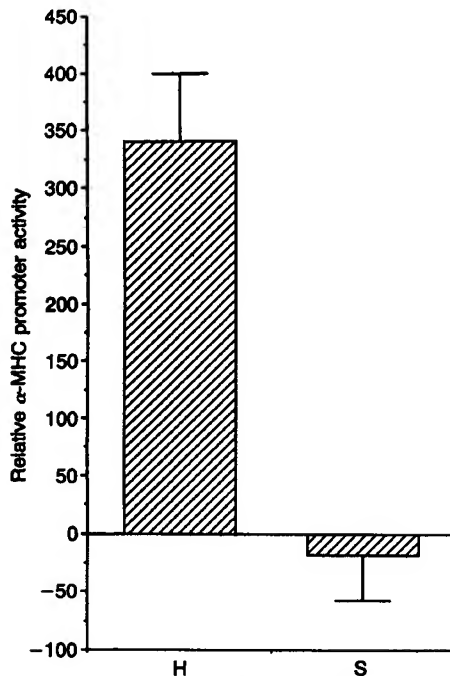


FIG. 2. Tissue-restricted expression of a gene injected into heart and skeletal muscles. Luciferase activities were normalized to CAT activities in DNA-injected hearts (bar H) and skeletal muscles (bar S) and expressed as relative α -MHC promoter activity [raw luminometer units (RLU)/% conversion]. Rat hearts and skeletal muscles were co-injected with each member of the following pairs of plasmids at 100 μ g: p α -MHCluc/pRSVCAT (n = 6 hearts and 12 skeletal muscles) and pXP2/pRSVCAT (n = 3 hearts and 11 skeletal muscles). Five days later, organ homogenates were assayed for activities of CAT (15% of each homogenate preincubated at 65°C for 10 min) and luciferase (5% of each homogenate). Only those muscles with CAT conversions >0.25% or approximately twice background (n = 6 hearts and 8 skeletal muscles injected with p α -MHCluc/pRSVCAT and 3 hearts and 10 skeletal muscles injected with pXP2/pRSVCAT) were chosen for analysis. Data are expressed as mean \pm SEM (P < 0.001).

skeletal muscle. These results demonstrate that α -MHC promoter directs detectable expression of a gene in a tissue-restricted manner.

Thyroid Hormone Modulation of Gene Expression. To assess the ability to modulate the expression of an injected gene

Table 1. Luciferase and CAT activities of hearts and skeletal muscles injected with p α -MHCluc and pRSVCAT

Organ	<i>n</i>	Luciferase activity, RLU			% CAT conversion		
		Mean	Range	SEM	Mean	Range	SEM
Heart	6	11,580	6,509–18,667	1975	38.6	18.8–88.7	10.7
Skeletal muscle	8	160	91–237	19	2.2	0.4–7.2	0.8

Each organ was co-injected with 100 μ g of each plasmid. Animals were sacrificed 5 days after the injection. Luciferase activity was measured in raw luminometer units (RLU) in 5% of the homogenate above the machine background which was measured in noninjected organs. Background resulting from the promoterless luciferase construct was determined by injecting 100 μ g of pXP2 and 100 μ g of pRSVCAT resulting in 139 RLU (range, 63–265 RLU; SEM, 64 RLU; n = 3) and 133 RLU (range, 177–236 RLU; SEM, 19 RLU; n = 10) in 5% of the homogenates from hearts and skeletal muscles, respectively. % CAT conversion in 15% of the homogenate above background was measured. CAT background was determined in 10 skeletal muscles injected with 100 μ g of p0-CAT (promoterless CAT construct) and 100 μ g of p α -MHCluc (mean, 0.14%; range, 0.07–0.51%; SEM, 0.04%).

as well as to determine the feasibility of mapping the regulatory elements of a gene, we studied the influence of thyroid hormone on the expression of injected p α -MHCluc DNA. α -MHC mRNA (10) and protein (24) are positively regulated by thyroid hormone *in vivo*. A construct bearing the same 5' flanking region of the rat α -MHC gene used in the current study has been shown to confer thyroid hormone responsiveness to a reporter gene transfected into fetal cardiac myocytes when normalized to the expression of a cotransfected RSV-driven β -galactosidase construct (12). However, the regulatory elements of this gene have not been mapped *in vivo* nor has it been possible to assess the thyroid responsiveness of DNA constructs in the adult heart. To assess the ability of p α -MHCluc to respond to thyroid hormone, animals were made hyperthyroid by two models and their hearts were injected with reporter genes.

In one model, three groups of adult female Wistar rats were co-injected with 100 μ g of p α -MHCluc and 100 μ g of pRSV-CAT: hypothyroid animals, hypothyroid animals treated with thyroid hormone, and euthyroid controls. Serum thyroxine was undetectable in the propylthiouracil-treated animals after the initial 3-week treatment interval. Further, these animals also had a flat growth curve as compared with controls, consistent with hypothyroidism. Hypothyroid animals treated with T3 had significant increases in heart weights consistent with induced hyperthyroidism. As a control, Northern blot hybridization of total RNA purified from the hearts of these animals (Fig. 3) demonstrated the expected changes in the expression of the endogenous MHC genes: a transition from α - to β -MHC mRNA in the hearts of hypothyroid animals compared with controls and a switch from β - to α -MHC expression in those of hypothyroid animals treated with T3 compared with animals remaining hypothyroid (10). These parameters confirmed that the various treatments resulted in the altered cardiac states associated with thyroid hormone manipulations.

RSV-driven CAT activity was depressed by factors of between 8 and 3 in two experiments in hypothyroid animals treated with T3 versus both hypothyroid and control groups (Fig. 4A; data not shown). Although the explanation for this phenomenon has not yet been elucidated, mixing studies excluded the presence of a specific inhibitor of CAT activity in T3-treated heart tissue (data not shown). When luciferase activity was normalized to CAT activity (Fig. 4B), the positive responsiveness of the α -MHC promoter to thyroid hormone was evident. The mean luciferase/CAT ratio of hypothyroid animals treated with T3 was approximately 3 times greater than that of the hypothyroid animals not treated with T3 (P < 0.005) and two times greater than that of euthyroid controls (P < 0.05).

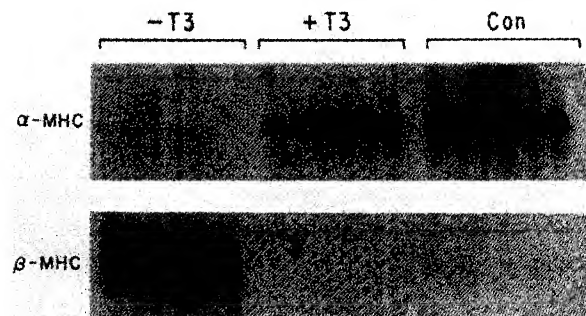


FIG. 3. Endogenous α - and β -MHC mRNA levels in hearts of thyroid-hormone-manipulated animals. Lanes -T3, +T3, and Con contain hypothyroid animals (n = 6), hypothyroid animals treated with T3 (n = 6), and euthyroid controls (n = 6), respectively. The above autoradiograms for α - and β -MHC resulted from 72- and 16-hr exposures, respectively, at -70°C with an intensifying screen.

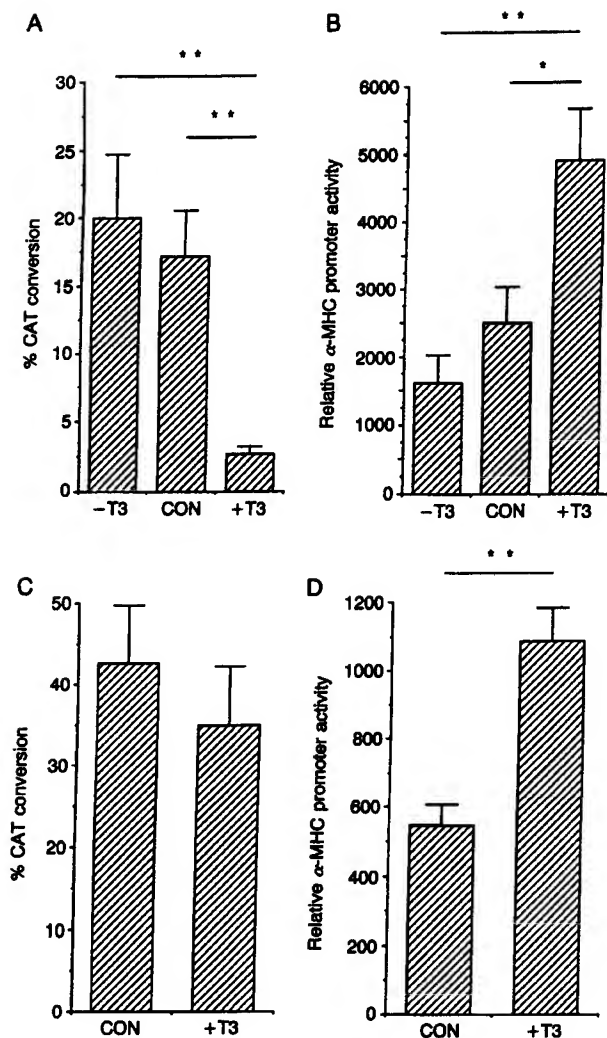


FIG. 4. Thyroid hormone modulation of the expression of a gene injected into heart muscle. Activities of CAT (% conversion) and luciferase [raw luminometer units (RLU)] in hearts co-injected with α -MHC-luc and pRSVCAT from thyroid-hormone-manipulated animals were measured. (A and B) CAT activity (A) (in 15% of the homogenate) and the relative α -MHC promoter activity (B) [luciferase/CAT ratio (in 5% of the homogenate/15% of the homogenate, respectively)] in hearts from hypothyroid animals (bars -T3; $n = 6$), hypothyroid animals treated with T3 (bars +T3; $n = 6$), and euthyroid controls (bars Con; $n = 5$) were measured. Homogenates were made from the same hearts as in Fig. 3. (C and D) CAT activity (C) (in 5% of the homogenate preincubated at 65°C for 10 min) and the relative α -MHC promoter activity (D) [luciferase/CAT ratio (in 5% of the homogenate/5% of the homogenate heated as above, respectively)] in hearts from euthyroid animals (bars Con; $n = 8$) and euthyroid animals treated with T3 (bars +T3; $n = 6$). Data are expressed as mean \pm SEM. *, $P < 0.05$; **, $P < 0.005$.

In the second protocol, hearts of euthyroid animals were co-injected with α -MHC-luc and pRSVCAT, after which half of the animals were treated with T3 as described above. Four days later, luciferase and CAT activities were determined in cardiac homogenates. As shown in Fig. 4C, RSV-driven CAT activities in the euthyroid and hyperthyroid hearts were not significantly different. Luciferase activity, when normalized to CAT activity (Fig. 4D), was significantly higher in the T3-treated animals ($P < 0.005$). The lower T3 responsiveness in the second of the two protocols (1.7 versus 3.0) is not surprising since euthyroid hearts are α -MHC predominant whereas hypothyroid hearts contain mostly β -MHC (see Fig.

3). These results coupled with the tissue-restricted nature of the α -MHC promoter strongly suggest that the cell type targeted for expression by the cardiac injections is the myocyte.

DISCUSSION

Our results demonstrate that the heart can be transfected *in vivo* by direct injection of a plasmid containing a viral or cellular promoter coupled to the coding sequence of a gene. Further, these experiments demonstrate that the expression of an injected gene spliced to a cellular promoter is regulated in a tissue-restricted and thyroid-hormone-responsive manner.

The amount of RSV-directed CAT activity per μ g of DNA injected into skeletal muscle in our experiments was similar to that observed previously (6). Given the 10- to 100-fold higher CAT activities detected in hearts as compared with skeletal muscles injected with pRSVCAT, the heart appears to be especially receptive to this type of manipulation. Why muscle in general and heart in particular expresses injected genes at significantly higher levels than do other tissues (6) is unclear. Although cell damage or inflammation may mediate gene transfer, the discordant responses in different tissues suggests a more fundamental mechanism. Both heart and skeletal muscle have rich transverse tubule systems allowing extensive contact between the myocytes and the interstitial space, and this may play a role as may the fact that both organs consist primarily of either bi- or multinucleated cells. It is also clear that the physiological status of the muscle may have profound effects on its ability to take up and/or express injected DNA (Fig. 4 A and C).

These studies demonstrate the feasibility of identifying the regulatory elements of genes expressed in cardiac muscle using the direct injection technique. This approach may obviate the need for the expensive and labor-intensive generation of transgenic animals for these purposes. Further, our results indicate that the 613 base pairs of the 5' flanking region of the cardiac α -MHC gene behaves the same in the intact heart and in transiently transfected cultured fetal myocytes with respect to thyroid hormone responsiveness (12). It is noteworthy that the α -MHC promoter was not negatively regulated in the hypothyroid animals, despite the fact that steady-state mRNA levels of α -MHC in the same animals were reduced by at least a factor of 5 relative to control animals (Fig. 3). Although the lack of a statistically significant reduction in promoter activity might have reflected the relatively small number of animals studied or the timing of sacrifice, this result suggests that posttranscriptional controls, perhaps influencing α -MHC mRNA half-life, are important in moderating the genetic response to this altered hormonal condition *in vivo*. A precedent for this type of posttranscriptional regulation has been seen with other hormones (25–27).

In summary, this report demonstrates that the heart is an excellent target organ for direct gene transfer and that cellular promoters transfected *in vivo* in this fashion behave in a tissue-restricted and physiologically responsive manner. The implications of this result for the study of cardiac physiology and disease are significant. By using this technique, it should be possible to map regions of cellular promoters responsive to complex stimuli that can be modeled only *in vivo*, such as hemodynamic overload and pathological states such as hypertension, conditions that are known to result in alterations in gene expression (7). In addition, constructs coupling specific genes to cellular promoters may be useful in targeting the expression of those genes to the heart and altering the physiology of the intact organ.

Note Added in Proof. While this manuscript was under review, two groups reported the expression of virally promoted reporter genes in rat hearts after direct DNA injection (28, 29).

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EXHIBIT O



A High-Titer Lentiviral Production System Mediates Efficient Transduction of Differentiated Cells Including Beating Cardiac Myocytes

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T. SAKODA, N. KASAHARA, Y. HAMAMORI AND L. KEDDES. A High-Titer Lentiviral Production System Mediates Efficient Transduction of Differentiated Cells Including Beating Cardiac Myocytes. *Journal of Molecular and Cellular Cardiology* (1999) 31, 2037–2047. Human immunodeficiency virus (HIV, lentivirus) type-1 based vectors have a number of attractive features for gene therapy, including the ability to transduce non-dividing cells and long term transgene expression. We used a three-plasmid expression system to generate pseudotyped lentivirus-based vectors by transient transfection of human embryonic kidney 293T cells in the presence of sodium butyrate, which is known to activate the long terminal repeat-directed expression of HIV. Using this system we successfully generated versatile high titer lentivirus at titers of up to 2×10^8 transducing units/ml (TU/ml), and improved transduction efficiency in various cell types from seven to over twenty fold. We demonstrate its applicability of these vectors for the efficient transduction of non-dividing cells, including post mitotic beating rat cardiac myocytes and well-differentiated rat L6 myofibers. While both lentivirus-based and murine retrovirus-based vectors efficiently transduced dividing cardiac fibroblasts and L6 muscle myoblasts in culture, lentivirus-based vectors also efficiently transduced cardiac myocytes and yielded titers of $(6.3 \pm 1.2) \times 10^5$ TU/ml; however murine retrovirus-based vectors showed low transduction efficiency with titers reaching only $(8.9 \pm 2.1) \times 10^2$ TU/ml. Furthermore, even 12 days after induction of differentiation of L6 myofibers, lentivirus-mediated transduction of β -galactosidase (β -Gal) at approximately 30–40% of the maximum expression levels achieved in replicating myoblasts. In contrast, the expression of β -Gal following transduction of the myofibers by murine retrovirus-based vectors fell to less than 1% of an already reduced level of transduction in undifferentiated confluent myoblasts. These results demonstrate that lentivirus-based vectors can efficiently transduce both well-differentiated cardiac myocytes and differentiated myofibers. This appears to be an efficient method and provides a new tool for research and therapy of cardiovascular diseases. © 1999 Academic Press

KEY WORDS: Lentivirus; Sodium butyrate; Cardiac myocyte; L6 cell; Vesicular stomatitis virus; Retrovirus; Gene therapy.

Introduction

Gene therapy has the potential to reverse the genetic causes and modify the pathophysiology of many innate and acquired diseases (Mulligan *et al.*, 1993;

Crystal *et al.*, 1995; Leiden *et al.*, 1995; Verma and Somia, 1997). Transduction of foreign DNA into cardiac myocytes or skeletal muscle myofibers is of potential value for therapeutic applications (Lacount *et al.*, 1996; Partridge and Davies, 1995) and also

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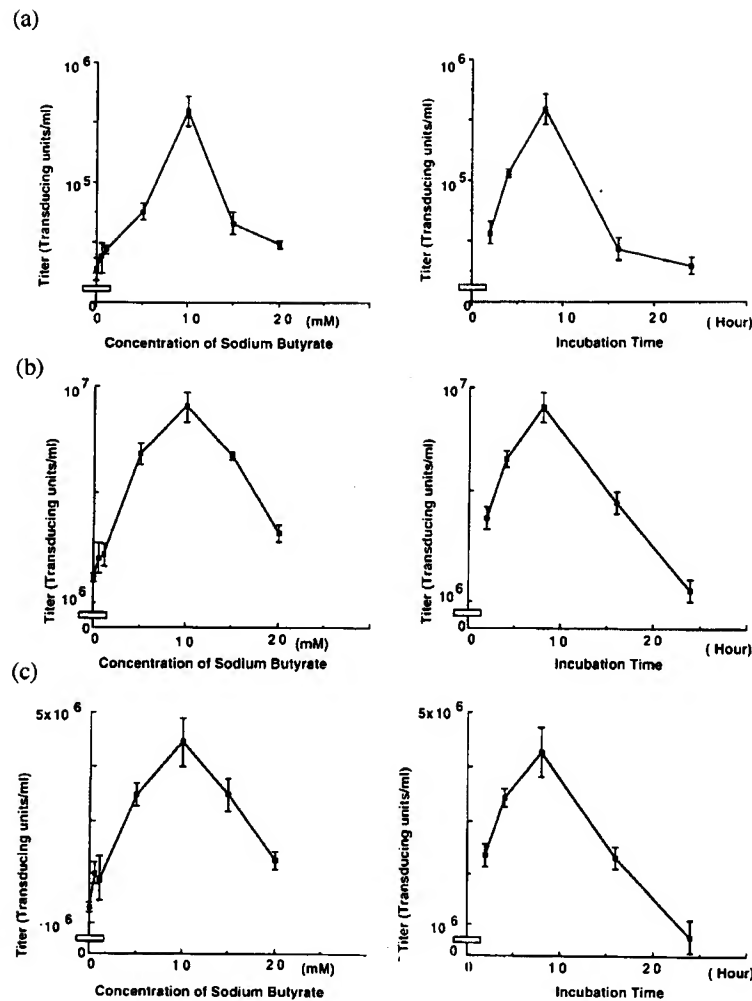


Figure 1 Effects of various concentrations and incubation times of sodium butyrate on titers (transducing units/ml, TU/ml) in various cells. (a) cardiac myocytes; (b) 293T cells; (c) L6 cells. Transduction is enhanced when 293T cells were exposed to sodium butyrate after transfection. Lentivirus-based vectors including GFP were generated by transient co-transfection of 293T cells. Sodium butyrate was administered at various concentrations and/or various intervals. After replacing with fresh medium without sodium butyrate, conditioned medium was harvested, filtrated, and used for vector preparation. The values are means \pm standard errors of three independent experiments.

offers an experimental approach to investigate the roles of individual genes in cardiovascular and muscle pathophysiology. Both efficient delivery and long-term expression of transduced genes is required before the full benefit of genetic manipulation strategies can be realized in the cardiovascular and muscular systems. However, current methods of gene delivery each suffer major limitations.

Non-viral methods of gene delivery remain inefficient and only attain transient expression of the transgene (Shi *et al.*, 1994). While adenovirus-based vectors allow highly efficient delivery of transgenes *in vitro* and *in vivo*, expression is transient

because the transgene is not integrated in the host genome and immune responses against the transduced cells remain a concern (Schulick *et al.*, 1995). In contrast, standard retrovirus-based vectors, such as the Moloney murine leukemia virus (Mo-MuLV), integrate the transgene into the genome of the target cells, which can sustain long-term expression. However, such retrovirus-based vectors can transduce only dividing cells (Lewis and Emerman, 1994), thus limiting their use in non-proliferating cells, especially differentiated cardiac myocytes and mature skeletal muscle myofibers. In addition, silencing of gene expression is a common

Table 1 Effect of optimum sodium butyrate exposure on lentiviral production

Cell Type	Sodium butyrate		Fold change
	(-)	(+)	
Cardiac Myocytes	$2.5 (\pm 1.1) \times 10^4$	$6.3 (\pm 1.2) \times 10^5$	25.2
L6 Myoblasts	$0.38 (\pm 0.1) \times 10^6$	$4.25 (\pm 0.5) \times 10^5$	11.2
293T Cells	$1.3 (\pm 0.3) \times 10^6$	$9.0 (\pm 0.5) \times 10^6$	6.9

Cells were exposed to medium with (+) or without (-) 10 mM sodium butyrate for 8 hours and lentivirus-based vectors were harvested 16 hours after switching to medium without sodium butyrate.

occurrence. Recently, development of lentivirus-based vector systems based on a modified retroviral HIV genome has provided a delivery system that can both transduce non-dividing cells and integrate the transgene in the genome of target cells (Naldini *et al.*, 1996a). In addition, such lentivirus-based vector particles can be pseudotyped with the envelope of the vesicular stomatitis virus (VSV) (Burns *et al.*, 1993), thus enabling the vector to introduce genes into a broad range of tissues (Naldini *et al.*, 1996a,b; Miyake *et al.*, 1998). These features of lentivirus-based vectors, including their lack of cytotoxicity, make them potentially useful for delivery of transgenes to cardiac myocytes and muscle myofibers. However there have been few reports regarding application of lentivirus-based vectors to cardiac myocytes and muscle myofibers (Rebolledo *et al.*, 1998; Kari *et al.*, 1997; Mochizuki *et al.*, 1998) and these reports do not address the transducibility of such cells at various stages of differentiation compared with conventional retrovirus-based vectors. In this study we compare the degree of efficiency of lentivirus-based vectors with that of murine retrovirus-based vectors for transduction of cultured primary rat cardiac myocytes, cardiac fibroblasts, rat L6 proliferating myoblasts and differentiated multinucleated myofibers. Modification of the lentiviral particle production system, enabled us to increase viral particle titers more than 10-fold using sodium butyrate and to routinely generate titers of $>10^8$ TU/ml after one round of concentration. The derived lentivirus-based vectors carrying reporter genes (bacterial β -Gal (LacZ) or jellyfish green fluorescent protein (GFP)) efficiently transduced not only proliferating rat cardiac fibroblasts and L6 myoblasts, but also non-dividing cardiac myocytes and well-differentiated L6 myofibers. In contrast, murine retrovirus-based vectors transduced only dividing cardiac fibroblasts and L6 myoblasts, but did not transduce non-proliferating cardiac myocytes or differentiated myofibers. These findings suggest that

lentivirus-based vectors should be useful for delivery of genes to the myocardium and skeletal muscle *in vivo*.

Materials and Methods

Cells and cell culture

Primary cultures of neonatal rat cardiac myocytes and cardiac fibroblasts were prepared as previously described (Sussman *et al.*, 1997; Ueyama *et al.*, 1997). The L6 rat skeletal muscle cell line was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Differentiation was initiated by replacing the medium with DMEM with 2% horse serum (HS) (Yarbee, 1968). 293T human kidney cells as well as producer cells of the Mo-MuLV-derived BAG retrovirus which bears the β -Gal gene, were grown in DMEM supplemented with 10% FCS in a 37°C, 5% carbon dioxide humidified environment (Naldini *et al.*, 1996a,b; Price *et al.*, 1987).

Virus production and transduction

Lentivirus-based vectors encoding β -Gal or GFP were generated by transient co-transfection of 293T cells with a three-plasmid combination, as described previously, with slight modifications (Naldini *et al.*, 1996b). Briefly, a 100 mm dish of non-confluent 293T cells were transfected with 15 μ g of pCMV Δ R8.2, 15 μ g of either pHR'-CMVLacZ or pHR'-CMVGFP and 15 μ g of pMDG by the calcium phosphate DNA precipitation method (Chen and Okayama, 1987; Sakoda *et al.*, 1992). The plasmid vectors were kindly provided by Dr Luigi Naldini (Cell Genesys Inc., Foster City, CA). Sixteen hours after transfection, the media was adjusted to a final concentration of 10 mM sodium butyrate and the

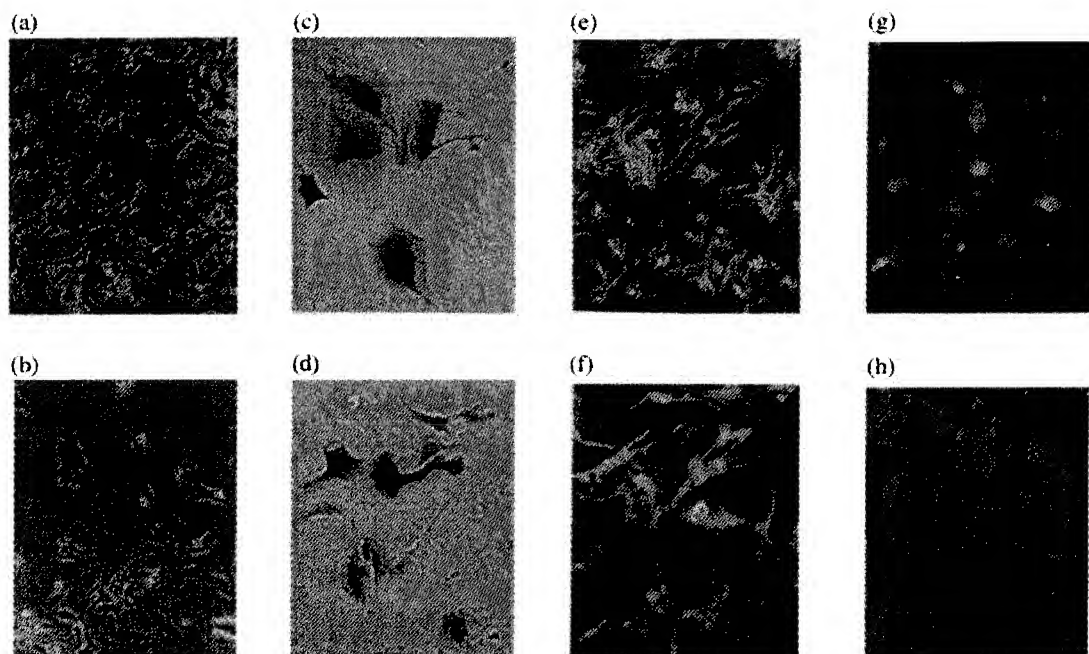


Figure 2

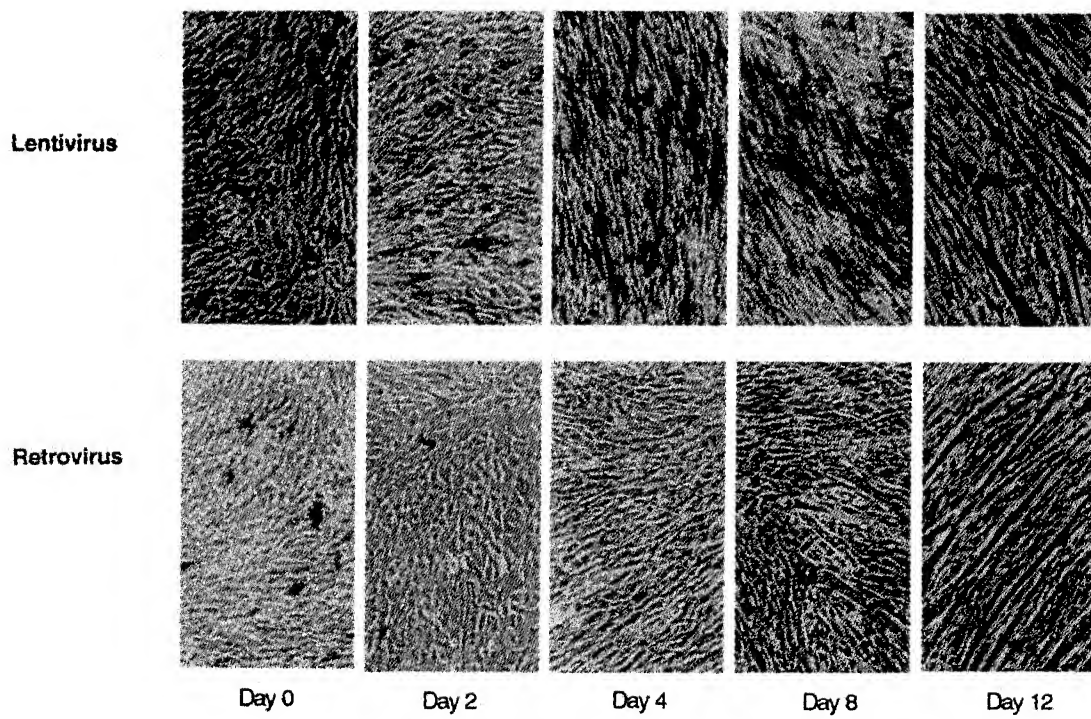


Figure 4

cells incubated for 8 hours unless noted otherwise in the text and figure legends. After the 8 hour incubation, cells were washed and incubated in fresh medium without sodium butyrate. Conditioned medium was harvested 16 hours later and filtrated through 0.45- μ m filters. Concentrated viral stocks were prepared by one-round of ultracentrifugation of 3 ml of the conditioned medium at 50 000 *g* at 4°C for 90 minutes in an SW41 rotor (Beckman Instruments). The pellet was resuspended overnight at 4°C in 30 μ l of Hanks balanced salt solution as described previously (Burns *et al.*, 1993). For transduction of cardiac myocytes, cardiac fibroblasts, L6 cells or 293T cells, cells were infected overnight with serial dilutions of virus stock in cultured medium supplemented with 8 μ g of polybrene per ml. After medium replacement, the cells were incubated for 48 hours and expression of β -Gal or GFP was assessed. BAG retrovirus-based vectors carrying the β -Gal gene were prepared by harvesting supernatants from a stable producer cell line (Price *et al.*, 1987). Transductions were performed in the presence of 8 μ g of polybrene per ml.

Analysis of transduced cells

Titers (TU/ml) were calculated by dividing the number of β -Gal or GFP expressing cells counted per dish by the dilution factor. Cells expressing β -Gal

figure 2 (opposite) β -Gal staining of cardiac myocytes and cardiac fibroblasts by lentivirus- or murine retrovirus-mediated β -Gal gene transduction, GFP fluorescence staining of cardiac myocytes by lentivirus-mediated GFP transduction and indirect immunofluorescence staining of cardiac myocytes using primary antisera against myosin (MF20). β -Gal, GFP fluorescence and indirect immunofluorescence staining were performed as described in Materials and Methods. (a) lentivirus-mediated β -Gal staining of cardiac myocytes; (b) murine retrovirus-mediated β -Gal staining of cardiac myocytes; (c) lentivirus-mediated β -Gal staining of cardiac fibroblasts; (d) murine retrovirus-mediated β -Gal staining of cardiac fibroblasts; (e) cardiac myocytes under bright field illumination—the same field as (c). (f) lentivirus-mediated GFP staining of cardiac myocytes; (g) lentivirus-mediated GFP staining of cardiac myocytes; (h) MF20 staining of cardiac myocytes—the same field as (g).

figure 4 (opposite) Immunohistochemical staining for β -Gal of L6 cells by lentivirus- or murine retrovirus-mediated β -Gal gene transduction. L6 cells were grown in DMEM supplemented with 10% FCS until they reached confluency, at this point the medium was replaced by DMEM supplemented with 2% HS for induction of differentiation. Cells were transduced at indicate days after replacing with differentiation medium.

were fixed and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) as described previously (Sanes *et al.*, 1986). Briefly, the cells were fixed with phosphate-buffered saline (PBS) containing 0.5% glutaraldehyde for 10 minutes at room temperature. After fixation, β -Gal expression was evaluated by histochemical staining with X-Gal in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 1 mM $MgCl_2$ and 1 mg/ml X-Gal at 37°C for 16 hours. β -gal activity was measured using a β -galactosidase Enzyme Assay System (Promega) as directed by the manufacturer. In brief, transduced cells were washed with PBS and lysed in Reporter Lysis Buffer. 150 μ l of these cell lysates were incubated with equal volume of Assay Buffer, which contains the substrate ONPG (o-nitrophenyl- β -D-galactopyranoside), for 30 minutes at 37°C. The absorbance of the samples at 420 nm was measured and β -Gal activities (μ U/ μ g protein) were determined by a calibration curve. Protein concentrations were determined by the method of Bradford with bovine serum albumin used as a standard protein (Bradford *et al.*, 1976).

Rat cardiac myocytes were stained with anti-myosin monoclonal antibody MF20 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) followed by Texas Red dye-conjugated goat anti-mouse immunoglobulin G (IgG). Briefly, isolated cardiac myocytes were fixed with 4% buffered formaldehyde for 5 minutes. The fixed cells were treated with 0.1% Triton X-100 for 2 minutes. After permeabilization in Triton X-100, cells were incubated for 1 hour with MF20, followed by incubation with Texas Red dye-conjugated goat anti-mouse IgG (1:333) for 1 hour at room temperature. Finally, the cardiac myocytes were washed several times in PBS and mounted on slides. Texas Red dye-conjugated anti-mouse IgG was purchased from Jackson Immuno Research Laboratories, Inc.

Results

Sodium butyrate increases production of lentivirus-based vectors

293T cells were transfected with pCMV Δ R8.2, pMDG and pHR'-CMVGFP to produce lentivirus-based vectors encoding GFP. To examine the effects of sodium butyrate on virus production, cells were exposed to sodium butyrate at various concentrations and times starting 16 hours after transfection. At the end of this treatment, cells were replaced with fresh medium without sodium butyrate and the medium harvested 16 hours later.

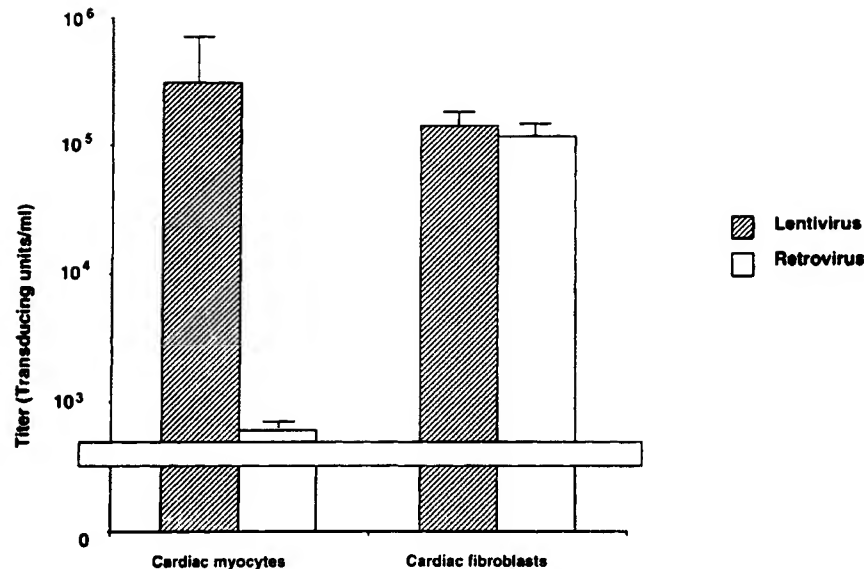


figure 3 Comparative efficiency of lentivirus- and murine retrovirus-mediated β -Gal gene transduction in cardiac myocytes and cardiac fibroblasts. Titers were determined as described under Materials and Methods. The values are means \pm standard errors of five independent experiments.

Figure 1 shows the effects of various concentrations and incubation times in sodium butyrate on the final viral vector titers. The viral titers were maximally enhanced when 293T cells were exposed to 10 mM sodium butyrate for 8 hours as determined by transduction of cardiac myocytes, 293T cells and L6 cells [Fig. 1(A–C)]. Sodium butyrate routinely increased titers in cardiac myocytes, 293T cells and L6 cells (Table 1). Thus, titers derived from 293T cells exposed to sodium butyrate were nearly one order of magnitude greater than those obtained without sodium butyrate. After only one round of concentration by ultracentrifugation at 50 000 *g*, titers of $(2.2 \pm 1.0) \times 10^8$ TU/ml on 293T cells were routinely achieved.

Lentivirus-, but not murine retrovirus-based vectors mediate high efficiency gene transfer into well differentiated rat neonatal cardiac myocytes

Highly efficient transfer and stable expression of genes delivered to the myocardium or skeletal muscle remains an elusive goal. To determine whether lentivirus-based vectors can efficiently transduce both well differentiated cardiac myocytes and skeletal muscle myofibers, we carried out a series of gene delivery experiments with cells in culture and contrasted the transduction efficiencies of lentivirus- and murine retrovirus-based vectors.

Primary neonatal rat cardiac myocytes and cardiac fibroblasts from the hearts of 2- to 3-day old rats were cultured for 3 days and then transduced with lentivirus-based or murine retrovirus-based vectors. The cells were stained with X-Gal or processed for immunofluorescence studies 3 days after the transduction. Figure 2(a) shows extensive β -Gal staining of cardiac myocytes by lentivirus-mediated LacZ transduction. While cardiac myocytes were successfully transduced by the lentivirus-based vectors, murine retrovirus-based vectors did not transduce the cardiac myocytes. The solitary β -Gal positive cell in Figure 2(b) is morphologically identified as a contaminating cardiac fibroblast. On the other hand, both lentivirus- and murine retrovirus-based vectors transduced cardiac fibroblasts [Figs 2(c) and (d) respectively]. Use of lentivirus-based vectors encoding GFP enabled us to assess transduction in living cells without fixation. Figure 2(e) and 2(f) show light and fluorescence confocal microscopy of such transduced cardiac myocytes. The GFP positive cells were beating cardiac myocytes. Finally, GFP positive cells clearly overlapped the Texas Red dye-positive myosin-expressing cells, indicating viral transduction of cardiac myocytes [Fig 2(g) and (h)]. The relative efficiencies of murine retrovirus- and lentivirus-based vectors on replicating cardiac fibroblasts and non-replicating cardiac myocytes are compared in Figure 3. In cardiac fibroblasts, both lentivirus- and murine retrovirus-

Table 2 Comparative efficiency of lentivirus- and retrovirus-mediated β -Gal transduction in L6 cells

Days after replaced to differentiation medium	Titer (transduction units/ml)	
	Lentivirus	Retrovirus
0	$1.45 (\pm 1.3) \times 10^6$ [100]	$8.95 (\pm 1.5) \times 10^5$ [100]
2	$8.53 (\pm 2.4) \times 10^5$ [58.8 \pm 16.6]	$9.40 (\pm 2.5) \times 10^4$ [10.5 \pm 2.79]
4	$6.63 (\pm 0.9) \times 10^5$ [45.7 \pm 6.21]	$6.01 (\pm 1.9) \times 10^4$ [6.72 \pm 2.12]
8	$5.96 (\pm 1.8) \times 10^5$ [41.1 \pm 12.4]	$4.28 (\pm 2.3) \times 10^3$ [0.48 \pm 0.26]
12	$5.25 (\pm 1.7) \times 10^5$ [36.2 \pm 11.7]	$6.32 (\pm 2.1) \times 10^2$ [0.07 \pm 0.02]

Cells were plated at low density and replaced by DMEM supplemented with 2% HS or induction of differentiation. Cells were transduced at indicated days after replacing with differentiation medium. Titers were determined as described under Materials and Methods. Results are also expressed relative to titer obtained in day 0. The values are means \pm standard errors of three independent experiments.

based vectors efficiently transduced and yielded similar titers of $(1.2 \pm 0.6) \times 10^5$ TU/ml and $(2.1 \pm 0.7) \times 10^5$ TU/ml, respectively. In cardiac myocytes, however only lentivirus-based vectors efficiently transduced with a titer of $(6.3 \pm 1.2) \times 10^5$ TU/ml. Furthermore, the GFP expression by lentivirus-based vectors persisted or at least 21 days with no apparent decrease (data not shown). The transduced cardiac myocytes showed no evidence of significant cytotoxicity by microscopic examination in line with previous reports (Naldini *et al.*, 1996b, Kari *et al.*, 1997). In contrast, murine retrovirus-based vectors showed low transduction efficiency with titers reaching only $(8.9 \pm 2.1) \times 10^2$ TU/ml. These results demonstrate that efficient long-term transduction of cardiac myocytes *in vitro* can be achieved by lentivirus-based, but not murine retrovirus-based vectors.

Lentivirus-, but not murine retrovirus-based vectors mediate high efficiency gene transfer into differentiated L6 rat skeletal myofibers

Murine retrovirus-based vectors can efficiently transduce proliferating myoblasts but not differentiated myofibers. We tested the hypothesis that, unlike the retrovirus-based vectors, lentivirus-based vectors would retain the ability to transduce skeletal muscle cells following differentiation either as low density myoblasts or as fused multinucleated myofibers.

To determine transduction titers of lentivirus- or murine retrovirus-based vectors in L6 cells, the cells were plated at a low density (1×10^5 cells/6 cm

dish) in DMEM supplemented with 10% FCS. The media was subsequently replaced by DMEM with 2% HS or induction of differentiation (Yarborough, 1968). Viruses were transduced at 0, 2, 4, 8 or 12 days after switching to differentiation medium. The cells were stained with X-Gal 3 days after the transduction. Table 2 shows the comparative efficiency of lentivirus- and murine retrovirus-mediated β -Gal gene transduction of the L6 cells. Results are also expressed as absolute titers as well as relative to titers obtained at day 0. When the cells were plated at a low density and switched to differentiation medium, they become elongated but not fused, and remained as distinctive individual cells even at day 12. Lentivirus-based vectors expressing β -Gal maintained a relatively high transduction efficiency even at day 12 ($36.2 \pm 11.7\%$), whereas the efficiency of transduction by murine retrovirus counterparts fell to only $0.07 \pm 0.02\%$.

We next compared the ability of lentivirus- and murine retrovirus-based vectors to transduce differentiated L6 myofibers. L6 cells were grown in DMEM supplemented with 10% FCS until they reached confluency. At this point, the medium was replaced with DMEM supplemented with 2% HS or induction of differentiation. Viruses were transduced at 0, 2, 4, 8 or 12 days after switching to differentiation medium. The cells were stained with X-Gal 3 days after transduction. As shown in Figure 4, lentivirus-based vectors can also efficiently transduce not only myoblasts (i.e. day 0) but also well differentiated myofibers (i.e. day 12). However, murine retrovirus-based vectors showed low transduction efficiency of myoblasts when confluent even at day 0. Moreover, in differentiated myofibers,

murine retrovirus-mediated β -Gal gene transduction was not observed. The comparative efficiency of lentivirus- and murine retrovirus-mediated β -Gal gene transduction in L6 cells is plotted in Figure 5.

Confluent L6 myoblasts transduced with lentivirus-based vectors encoding β -gal produced $188 \pm 2.3 \mu\text{U}/\mu\text{g}$ protein at day 0 and maintained 30–40% of this level ($69 \pm 10.6 \mu\text{U}/\mu\text{g}$ protein) even when transduced at day 12. However, the expression of β -Gal following transduction by murine retrovirus-based vectors was only $37.5 \pm 5.1 \mu\text{U}/\mu\text{g}$ protein at day 0 and fell to less than 1% ($0.31 \pm 0.67 \mu\text{U}/\mu\text{g}$ protein) at day 12 after differentiation. These results indicate that lentivirus-based vectors can efficiently transduce muscle cells at all stages of differentiation.

Discussion

We have developed an efficient system to enhance production of lentivirus-based vectors using sodium butyrate. Although much has been learned about the action of sodium butyrate upon cells, the molecular mechanisms of these effects remain unclear. Sodium butyrate produces a wide variety of effects on cells including the inhibition of histone deacetylase. Active transcription complexes or a modified chromatin structure, possibly via sodium butyrate-mediated acetylation of cellular histones has been suggested (Roman, 1982). There is considerable evidence for the enrichment of acetylated histones in transcriptionally active chromatin (Weisbrod, 1982). Accumulation of highly acetylated histones are required for efficient transcription (Grunstein, 1997).

With respect to the effect of sodium butyrate on virus production, sodium butyrate treatment has been reported to stimulate human cytomegalovirus (CMV) gene expression and viral replication in human endothelial cells (Radsak *et al.*, 1989). This enhancing effect by sodium butyrate on virus replication is not specific for human CMV. Similar results have been obtained using other human herpesviruses, Epstein-Barr virus (Saemundsen *et al.*, 1980) and herpes simplex virus (Ash, 1986). In addition, sodium butyrate has been reported to activate the long terminal repeat-directed expression of HIV (Bohan *et al.*, 1987). Therefore, sodium butyrate appears to be associated with a general induction of viruses. Whether these effects operate through some host cellular factors more efficiently transcribed when histones are highly

acetylated or the acetylation of host transcription factors themselves remains unknown.

In this study, we analysed the effect of sodium butyrate on production of lentivirus-based vectors. Sodium butyrate treatment at an optimal concentration of 10 mM or an optimal duration of 8 hours increased titers by about 9-fold in 293T cells, 11-fold in L6 cells and 25-fold in cardiac myocytes compared to those obtained without sodium butyrate (Fig. 1 and Table 1). Thus, sodium butyrate clearly enhanced viral titers and increased the efficiency of transduction in all cell types tested. In these experiments, we harvested viruses from conditioned medium 16 hours after sodium butyrate withdrawal. Though the half-life of lentiviral particles under these conditions is only 4–6 hours, the effect of sodium butyrate on virus production was continued after its withdrawal. It has been reported that sodium butyrate-activated transcriptional state is continued after withdrawing sodium butyrate from the culture medium and can be imprinted in daughter cells for many generations (Tang and Taylor, 1992). Sodium butyrate induction of the Moloney murine sarcoma virus enhancer promoter-induced transcription was shown to be propagated from mother to daughter cells after withdrawal of sodium butyrate.

The present study also demonstrates that lentivirus-based vectors can efficiently transfer and express transgenes in beating cardiac myocytes and differentiated L6 myofibers. Skeletal muscle is an excellent target for somatic cell gene therapy. Transgene expression in skeletal muscle can be easily accessed and its large mass of muscle allows repeated administration of the recombinant vectors. Introduction of genetic material into muscle fibers has become of great interest not only for its potential therapeutic values for genetic diseases of muscle such as Duchenne muscular dystrophy or myotonic dystrophy, but also for the conversion of skeletal muscle to the production of systematically active gene products unrelated to muscle function (Hamamori *et al.*, 1995). *In vivo*, skeletal muscle contains a mixture of myogenic cell types, including muscle stem cells (satellite cells), proliferative myoblasts and differentiated myofibers, whose relative numbers vary depending on the state of muscle maturity or processes such as repair or regeneration after injury. Therefore, gene transfer into muscle necessitates transduction at various stages of cell growth and differentiation with high efficiency and low toxicity. Cardiac myocytes on the other hand, are permanently withdrawn from the cell cycle soon after birth. Subsequently, myocardial growth is accomplished primarily by enlargement of

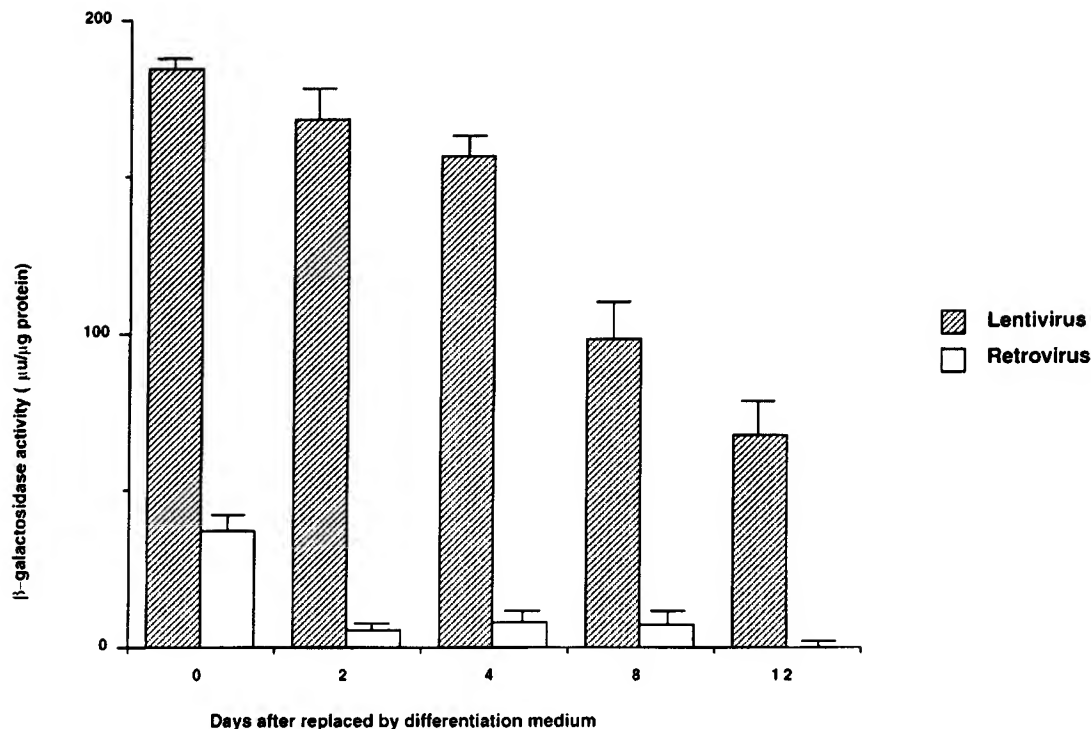


figure 5 Comparative efficiency of lentivirus- and murine retrovirus-mediated β -Gal gene transduction in L6 cells. Cells were grown in DMEM supplemented with 10% FCS until they reached confluency, at this point the medium was replaced by DMEM supplemented with 2% HS or induction of differentiation. Viruses were transduced at the indicated days after induction of differentiation. As assessment of transduced cell counts was difficult with used myofibers, cell lysates were prepared and β -Gal activity assayed 3 days after transduction. The results are expressed as β -Gal activity, μ U/ μ g protein. The values are means \pm standard errors of three independent experiments.

individual cardiac myocytes (i.e. hypertrophy), not by hyperplasia. Loss of cell in the myocardium following injury such as ischemia or cardiomyopathy, is not compensated by cell proliferation. Subsequently loss of myocardial mass can be a major cause of heart failure. For these reasons, or gene transfer to offer therapeutic potential to improve and maintain cardiac function, it cannot be accompanied by additional toxicity of the residual viable muscle into the heart, which cannot be well tolerated, but will rely on long-term expression with high efficiency.

Impressive gains have been made in delivery of genes to cardiac and skeletal myocytes using a variety of vectors. Although adenovirus-based vectors have been shown to be capable of transducing muscle cells *in vivo* and *in vitro* (Quantin *et al.*, 1990), gene expression was transient. In addition, standard E1, E3-deleted adenovirus-based vectors may have some direct cytopathic effects and induce immunological responses to transduced cells, because leaky expression of adenoviral genes can not

be completely eliminated (Schulick *et al.*, 1995). Retrovirus-based vectors derived from Mo-MuLV are the most commonly used vectors in current clinical human gene therapy trials (Mulligan, 1993). Retroviruses integrate into chromosomal DNA thus providing potential for long-term expression of the transduced gene. However, it has been demonstrated that passage of target cells through mitosis is required for efficient transduction (Lewis and Emerman, 1994). This requirement for cell division greatly limits the use of murine retrovirus-based vectors for therapeutic gene transfer, especially in non-replicating, differentiated cardiac and skeletal muscle myocytes. As an attractive alternative, lentivirus-based vectors pseudotyped with the VSV envelope have recently been reported to transduce a variety of cell types and this vector system also can transduce both dividing and non-dividing cells (Naldini *et al.*, 1996a,b). Furthermore, cytopathic or immunogenic effects of lentivirus-based vector itself have not been reported. In this study, we examined the transduction efficiency of

lentivirus-based vectors dividing and non-dividing cells and compared with murine retrovirus-based vectors. Our results show that unlike retrovirus-based vectors, lentivirus-based vectors can transduce genes efficiently into non-dividing cardiac myocytes and L6 myofibers at various stages of differentiation. As lentivirus-based vectors also integrate in the genome of the target cells (Naldini *et al.*, 1996a,b; Miyake *et al.*, 1998), repeated transduction is unnecessary. In consequence, humoral responses to repeat injections of viral antigens can be avoided (Knowles *et al.*, 1995), in contrast to adenovirus-based vectors. Moreover, the lentivirus-based vectors studied in this paper are completely replication-defective (Naldini *et al.*, 1996a), consequently, the transduced cells will not express viral proteins that might trigger cellular immune responses. The *in vitro* results from the present study provide important information for *in vivo* application of lentivirus-based vectors to cardiac and skeletal muscle. The availability of integrating vectors that can deliver genes to heart and muscle without evoking immune responses sets the stage for further studies of cardiovascular and neuromuscular gene therapy.

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Results

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EXHIBIT P

High-Titer Human Immunodeficiency Virus Type 1-Based Vector Systems for Gene Delivery into Nondividing Cells

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Previously we designed novel pseudotyped high-titer replication defective human immunodeficiency virus type 1 (HIV-1) vectors to deliver genes into nondividing cells (J. Reiser, G. Harmison, S. Kluepfel-Stahl, R. O. Brady, S. Karlsson, and M. Schubert, *Proc. Natl. Acad. Sci. USA* 93:15266–15271, 1996). Since then we have made several improvements with respect to the safety, flexibility, and efficiency of the vector system. A three-plasmid expression system is used to generate pseudotyped HIV-1 particles by transient transfection of human embryonic kidney 293T cells with a defective packaging construct, a plasmid coding for a heterologous envelope (Env) protein, and a vector construct harboring a reporter gene such as *neo*, *ShlacZ* (encoding a phleomycin resistance/ β -galactosidase fusion protein), *HSA* (encoding mouse heat-stable antigen), or *EGFP* (encoding enhanced green fluorescent protein). The packaging constructs lack functional Vif, Vpr, and Vpu proteins and/or a large portion of the Env coding region as well as the 5' and 3' long terminal repeats, the Nef function, and the presumed packaging signal. Using G418 selection, we routinely obtained vector particles pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G) with titers of up to 8×10^7 CFU/ μ g of p24, provided that a functional Tat coding region was present in the vector. Vector constructs lacking a functional Tat protein yielded titers of around 4×10^6 to 8×10^6 CFU/ μ g of p24. Packaging constructs with a mutation within the integrase (IN) core domain profoundly affected colony formation and expression of the reporter genes, indicating that a functional IN protein is required for efficient transduction. We explored the abilities of other Env proteins to allow formation of pseudotyped HIV-1 particles. The rabies virus and Mokola virus G proteins yielded high-titer infectious pseudotypes, while the human foamy virus Env protein did not. Using the improved vector system, we successfully transduced contact-inhibited primary human skin fibroblasts and postmitotic rat cerebellar neurons and cardiac myocytes, a process not affected by the lack of the accessory proteins.

Vectors based on oncoretroviruses such as Moloney murine leukemia virus (MoMLV) are useful to deliver therapeutic genes into primary cells in vitro and have also been applied in a number of gene marking and gene therapy trials with humans (13, 62). The principal advantages of retroviral vectors include the high efficiency of gene delivery, integration into the host cell genome, and high level of gene expression. One drawback of oncoretroviruses is their dependence on cell proliferation for completion of the life cycle (35, 56). Breakdown of the nuclear envelope that accompanies mitosis appears to be essential for the import of the viral preintegration complex into the nucleus and its integration into the genome of the host cell (26, 27, 51). In contrast, lentiviruses, including human immunodeficiency virus type 1 (HIV-1), differ fundamentally from oncoretroviruses in that they are independent of cell division for completion of their replicative cycle (58). This is an attractive feature in view of the need for vectors for nondividing cells such as neurons (14, 25). In common with all replication-competent retroviruses, the HIV-1 genome contains the *gag*, *pol*, and *env* coding regions, which encode the core proteins, the virion-associated enzymes, and the envelope (Env) glycoprotein, respectively, flanked by the long terminal repeats (LTRs). The LTRs include *cis*-acting sequences required for

integration, transcription, and polyadenylation. HIV-1 also possesses regulatory functions encoded by the *tat* and *rev* genes as well as accessory genes that include *vif*, *vpr*, *vpu*, and *nef*, many of which are not required for virus replication in vitro (17).

Another advantage of HIV-1 and other retroviruses is the fact that they can be pseudotyped by the incorporation of heterologous glycoproteins, allowing an extension of the host range of such vectors beyond cells expressing CD4. Several studies have demonstrated that HIV-1 produced in cells infected with xenotropic murine leukemia virus (6, 30), amphotropic murine leukemia virus (9, 55), or herpes simplex virus (64) gave rise to phenotypically mixed virions with an expanded host range, suggesting that pseudotyped virions had formed. Additionally, phenotypic mixing of viral envelopes was shown to occur between HIV-1 and vesicular stomatitis virus (VSV) in coinfecting cell cultures (64). Page et al. (40) showed that expression of amphotropic or ecotropic MoMLV Env glycoproteins in cells transfected with an HIV-1 vector construct produced virus capable of infecting both human and murine cells, and Landau et al. (23) demonstrated that HIV-1 efficiently incorporated the human T-cell leukemia virus type 1 Env. These observations were confirmed and extended by results showing that the VSV G glycoprotein (VSV-G) was efficiently incorporated into HIV-1 virions, with pseudotyped viral titers reaching 10^7 CFU/ml or higher (2, 38, 49).

A number of replication-defective HIV-1 vector systems have been described. With the original transient two-plasmid

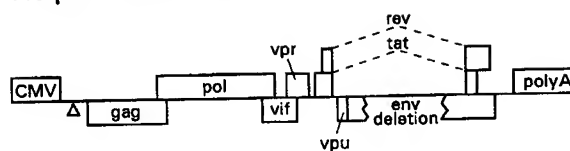
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expression system (23, 40), titers of up to 2×10^5 CFU/ml were obtained on human osteosarcoma (HOS) cells. Several groups have designed transient three-component HIV-1 expression systems consisting of a packaging construct, a plasmid bearing the gene encoding gp160, and an expression vector carrying a reporter gene (5, 41, 42, 45, 50, 53, 59), thus reducing the likelihood of generating replication-competent virus. In general, these systems were quite inefficient, with titers of around 10^4 CFU/ml or below. A number of studies have dealt with the design of HIV-1-based packaging cell lines. While the initial vector titers were quite low (7), recent improvements which involve tetracycline-controlled HIV-1-based packaging constructs (63), different molecular clones (11, 44), or Rev-independent cell lines (57) suggest that the generation of high-titer HIV-1-based packaging cell lines will eventually be feasible. Improved three-component split packaging systems were recently described by Naldini et al. (38) and Kim et al. (21). In these systems, the viral particles were pseudotyped with the envelope of VSV. Titers of up to 9×10^5 transducing units per ml were obtained. In this report, we describe an improved and versatile high-titer three-plasmid-based packaging system and demonstrate its applicability for the efficient transduction of nondividing cells, including growth-arrested HOS cells, confluent primary human skin fibroblasts (HSFs), and postmitotic rat cardiac myocytes and cerebellar neurons.

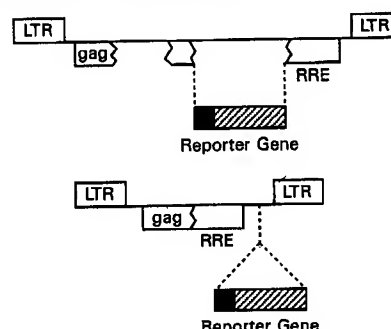
MATERIALS AND METHODS

Plasmid constructs. The following plasmids were obtained through the AIDS Research and Reference Reagents Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Md.: pHIVgpt and pHXB2-env from Kathleen Page and Dan Littman (40); pNL4-3 from Malcolm Martin (1); and p210-13, p210-19, and p210-25 from Ronald Desrosiers (17). All nucleotides are numbered according to the work of Myers et al. (37). The HIV-neo Δ vector constructs were derived from the HXB2 molecular clone (48). They harbor deletions from the *SpeI* site (nucleotide 1506) to the *EcoRI* (nucleotide 5742), *SalI* (nucleotide 5784), and *NdeI* (nucleotide 6399) sites. The plasmids are referred to here as pHIV-neo Δ E, pHIV-neo Δ S, and pHIV-neo Δ N, respectively. All three plasmids contain a truncated gp160 coding region with the sequences from the *NdeI* site at position 6399 to the *BglII* site at position 7611 deleted. They also contain a 168-bp fragment carrying the simian virus 40 (SV40) origin of replication (49). Plasmid HIV-neo Δ Tat (-) contains two consecutive termination codons after amino acid 10 within the 5' *tat* exon. It is based on pTat(-)GV/4GSTm (19), which was kindly provided by K.-T. Jeang (NIAID). Plasmid NL-neo is based on the NL4-3 molecular clone and carries a deletion from the *NsiI* site (position 1246) to the *BglII* site at position 7611. A 1,169-bp fragment carrying the *neo* gene sequence and the SV40 early promoter derived from pBK-CMV (Stratagene) was inserted between the *BamHI* site (nucleotide 8464) and *XhoI* site (nucleotide 8886). Plasmid HIV-HSA Δ E was derived from pHIV-HSA (49) by deleting the sequences between the *SpeI* (nucleotide 1506) and *EcoRI* (nucleotide 5742) sites. Plasmids HIV-Shl Δ CE and HIV-EGFP Δ E are based on HIV-HSA Δ E. The HSA (which encodes mouse heat-stable antigen) coding region was replaced with the *Shl* Δ C sequence derived from pUT535 (CAYLA, Toulouse, France), which codes for a bifunctional phleomycin/ β -galactosidase fusion protein (3), or EGFP (which encodes enhanced green fluorescent protein) sequences derived from pEGFP-C1 (Clontech). All Env-encoding plasmids except pHXB2-env are based on pLTR-G (49). Plasmids encoding the rabies virus and Mokola virus G proteins (33) were kindly provided by Karl-Klaus Conzelmann (Federal Research Centre for Virus Disease of Animals, Tübingen, Germany). The human foamy virus (HFV) *env* coding region (16) was kindly provided by Axel Rethwilm (University of Würzburg, Würzburg, Germany), and the MoMLV 4070A Env-encoding plasmid (39) was from Alan Rein (National Cancer Institute, Frederick, Md.). In the C-Help packaging construct, the 5' LTR was replaced with the human cytomegalovirus (CMV) immediate-early promoter (4). HIV-1 sequences from nucleotide 675 up to the *ApaI* site (nucleotide 2005) and sequences from the *SalI* site (nucleotide 5784) to the *XhoI* site (nucleotide 8886) were derived from the BH10 molecular clone (46). All other sequences were from the NL4-3 molecular clone. Plasmid C-Help carries a 1,212-bp *env* deletion (nucleotides 6399 to 7611) and an SV40 origin of replication (49). A 33-bp deletion harboring the putative packaging signal from nucleotides 756 to 789 between the 5' major splice donor site and the beginning of the *gag* coding region was introduced. Sequences distal to the *XhoI* site (position 8886) were removed and replaced with the bovine growth hormone polyadenylation site. A helper construct with a mutated *vpr* coding region was made by Klenow fill-in of *EcoRI*-digested C-Help plasmid DNA. A helper plasmid encoding a defective IN (integrase) protein was designed by replacing

A. Helper Constructs



B. Vector Constructs



C. Envelope Constructs



FIG. 1. Components of the HIV-1 packaging system. (A) Helper constructs. The open triangle symbolizes a 33-bp deletion affecting the packaging signal between the 5' splice donor site and the beginning of the *gag* sequence. Boxes interrupted by jagged lines contain partial deletions. (B) Transducing vector constructs. Top, vectors with expression cassette 5' of the RRE; bottom, vectors with expression cassette 3' of the RRE. (C) Env expression constructs.

the *ApaI-SalI* fragment (nucleotides 2005 to 5784) with the corresponding fragment from the D116N/7 molecular clone (15) (kindly provided by George Englund, NIAID). The C-Help Δ vpr plasmid was constructed by replacing the *ApaI-SalI* fragment with the corresponding fragment from plasmid p210-19 (17). C-Help Δ vif Δ vpr Δ vpu harbors sequences from p210-25 (*ApaI-SalI* fragment) and p210-13 (*SalI-NdeI* fragment) (17).

Plasmids pHIT60 and pHIT111 (54) were kindly provided by Alan Kingsman, Oxford University, Oxford, England. pCMV-G is based on pcDNA3.1/Zeo (Invitrogen) and harbors a 1.6-kb fragment encoding VSV-G. pG1-HSA is a MoMLV-based vector encoding mouse HSA.

Cells. Human embryonic kidney 293T cells (12) were kindly provided by Warren Pear (Rockefeller University). HOS cells (CRL-1543), Rat-2 cells (CRL-1764), and primary HSFs (CRL-2072) were obtained from the American Type Culture Collection. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc.) containing 10% heat-inactivated fetal bovine serum (FBS). Neonatal ventricular myocytes were harvested from the hearts of 2- to 3-day-old Sprague-Dawley rats and cultured as described previously (46). Cerebellar granule cells from 8-day-old Sprague-Dawley rat pups (Taconic Farms) were prepared and cultured as described by Taniwaki et al. (60). The cells were plated in poly-L-lysine-coated 35-mm-diameter dishes. After 1 day in culture, cytosine arabinoside (final concentration, 10 μ M) was added to the cells.

Virus production and infection. For the preparation of HIV-1 pseudotypes, helper plasmid DNA (5 μ g), Env plasmid DNA (5 μ g), and vector plasmid DNA (5 μ g) were cotransfected into subconfluent 293T cells by the calcium phosphate precipitation method (43). Approximately 2×10^6 cells were seeded into six-well plates 24 to 30 h prior to transfection. Chloroquine (25 μ M, final concentration) was added to the cells immediately before transfection, and the medium was replaced with fresh DMEM-10% FBS (2 ml per well) 12 to 14 h later. MoMLV-based virus stocks were generated by transient cotransfection of pHIT60, pCMV-G, and pHIT111 or pG1-HSA, respectively. The virus stocks were harvested 60 to 65 h posttransfection and filtered through a 0.45- μ m-pore-size filter, aliquoted, and subsequently frozen at -80°C . Target cells were infected in DMEM-10% FBS containing Polybrene (8 μ g/ml) for 3 to 8 h. The medium was subsequently replaced with fresh DMEM-10% FBS or preconditioned medium

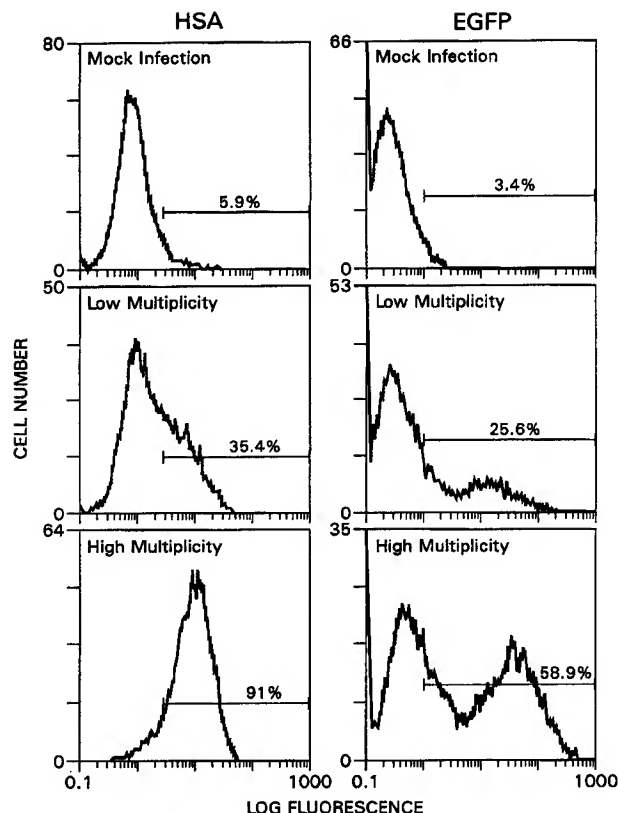


FIG. 2. Efficiency of *HSA* and *EGFP* reporter gene expression in HOS cells. Cells were infected with HIV-HSAΔE or HIV-EGFPΔE pseudotypes and tested for expression of the reporter genes by FACS analysis. Approximately 5×10^5 cells were infected with 0.4 ml of a control virus supernatant for the mock infection; for the low- and high-multiplicity infections, 0.04 or 0.4 ml, respectively, of HIV-HSAΔE or HIV-EGFPΔE pseudotype stock were used. Infected cells were processed for FACS analysis 3 days later.

for the cerebellar granule cells and heart ventricular myocytes. p24 assays were performed with a commercial kit (Cellular Products Inc.).

Analysis of transduced cells. Cells expressing HSA were detached from the plate by using phosphate-buffered saline–2 mM EDTA and stained with a fluorescein isothiocyanate-labeled anti-HSA monoclonal antibody (Caltag) for 30 min on ice in Hanks' balanced salt solution (Life Technologies) containing 2% FBS (Hanks'-FBS). The cells were washed twice with Hanks'-FBS, resuspended in 4% paraformaldehyde, and then subjected to fluorescence-activated cell sorting (FACS) analysis. Cells expressing EGFP were collected for FACS analysis as described above except that the paraformaldehyde step was omitted. Alternatively, they were analyzed by fluorescence microscopy. Cells expressing β-gal-

tosidase were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as described elsewhere (22). Rat cardiac myocytes were stained with the antimyosin monoclonal antibody MF 20 followed by rhodamine-labeled anti-mouse immunoglobulin G. Virus titers were determined by limiting dilution. Target cells were split into six-well plates the day prior to infection to give approximately 50% confluence at the time of infection. Infections were performed with serial dilutions of virus stock in a total of 0.5 ml of medium containing 8 μg of Polybrene per ml. After 3 to 6 h at 37°C, 2 ml of medium was added and the plates were incubated at 37°C for an additional 3 days. The medium was then aspirated, and 2 ml of medium supplemented with G418 (0.35 to 0.5 mg of active drug per ml; Life Technologies) was placed into each well. The medium was changed every 3 to 4 days, and the colonies were counted on day 10 or 14 after staining with crystal violet (0.2% in 20% ethanol). Alternatively, infected cells were trypsinized 3 days after infection and serially diluted into DMEM-FBS containing G418, and the colonies were stained 10 days later (23). The final yield of colonies was corrected for the increase in cell number between the time of infection and selection.

RESULTS

Design of an improved HIV-1-based vector system. Our original two-component HIV-1-based vector system was composed of a vector construct and an independent Env-encoding component (49). The vector construct carried a deletion within the *env* coding region and harbored a reporter gene to be transferred to the target cells. It contained all sequences necessary for reverse transcription, vector integration, and expression of the reporter gene. In one of the vectors, an expression cassette consisting of the SV40 early promoter driving the bacterial *neo* gene was used. An additional vector contained the mouse *HSA* coding region as a reporter gene under the control of the human CMV immediate-early promoter. The formation of replication-competent HIV-1 was precluded because a substantial portion of the *env* coding region was missing in these vectors.

We have now improved this original vector system in terms of safety and flexibility. To minimize further the generation of replication-competent virus, the original two-component system was split into three components: a helper construct, a vector component, and an Env-encoding plasmid (Fig. 1). Our helper constructs (Fig. 1A) express the Gag, Pol, Tat, and Rev functions and, depending on the construct, retain functional *vif*, *vpr*, and *vpu* genes, but Nef is always absent. The 5' LTR was replaced by the human CMV immediate-early promoter, and a heterologous polyadenylation signal was used instead of the 3' LTR. All helper plasmids lack *cis*-acting sequences that have been implicated as important for efficient HIV-1 RNA packaging (10, 24, 32). The vector constructs (Fig. 1B) contain a reporter gene such as *neo*, *HSA*, *ShlacZ*, or *EGFP*. They also contain *cis*-acting sequences required for packaging, reverse transcription, and integration, including the 5' and 3' LTRs, and *env*-derived sequences encompassing the Rev response element (RRE). The expression cassette was placed either

TABLE 1. Effect of packaging construct on pseudotype formation^a

Packaging construct	Functions missing	Virus titer (CFU/μg of p24) ^c	Relative HSA expression ^d (% of C-Help level)
None	NA ^b	<10 ^{2d}	<1
C-Help	Ψ, Env, Nef, 5' LTR, 3' LTR	5.8 × 10 ⁷	100
C-Help vpr ⁻	Ψ, Vpr, Env, Nef, 5' LTR, 3' LTR	5.0 × 10 ⁷	153
C-Help Δvpr	Ψ, Vpr, Env, Nef, 5' LTR, 3' LTR	4.6 × 10 ⁷	144
C-Help ΔvifΔvprΔvpu	Ψ, Vif, Vpr, Vpu, Env, Nef, 5' LTR, 3' LTR	8.5 × 10 ⁷	109
C-Help IN	Ψ, Int, Env, Nef, 5' LTR, 3' LTR	5.3 × 10 ⁴	<1

^a Vector constructs used were HIV-neoΔE and HIV-HSAΔE; the Env construct used encoded VSV-G.

^b NA, not applicable.

^c Determined by endpoint dilution ($n = 3$). Cells were selected in medium containing G418. The colonies were stained 10 days later.

^d <10² indicates that no colonies were obtained above the detection limit of the assay.

^e Determined by FACS analysis ($n = 3$). <1 indicates signal at background level.

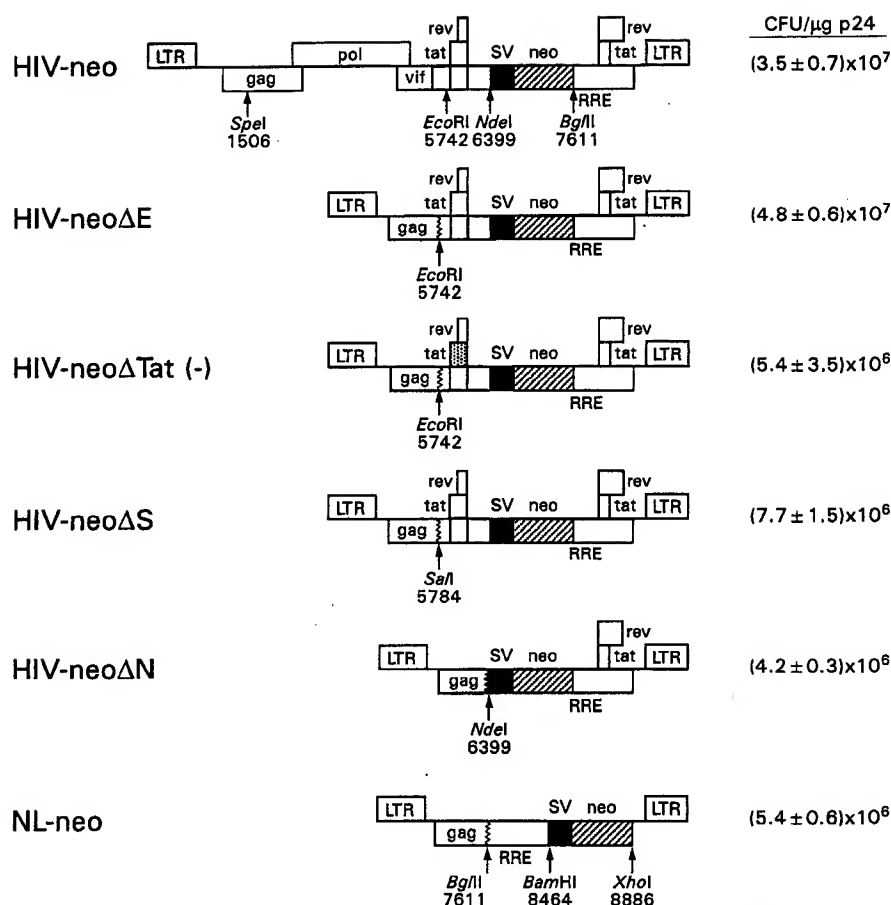


FIG. 3. Influence of Tat in vector construct on efficiency of formation of G418-resistant colonies. The top portion represents the original HIV-neo vector (49). The various deletion derivatives are shown below. The point-mutated 5' *tat* exon in HIV-neoΔTat (-) is highlighted with dots. NL-neo carries the *neo* expression cassette 3' of the RRE. The efficiency of colony formation (average \pm standard deviation of the results of three to four independent experiments) is shown on the right for each vector construct.

upstream or downstream of the RRE, depending on the design of the vector. The envelope constructs (Fig. 1C) provide a heterologous Env protein that leads to formation of HIV-1 pseudotypes. The Env proteins that were tested were those of VSV, rabies virus, Mokola virus, MoMLV, and HFV.

The ability of the newly designed vector system to mediate gene transfer was initially analyzed by infecting HOS cells with pseudotyped vectors carrying the *HSA* or *EGFP* reporter gene. Virus stocks were generated by transient cotransfection of 293T cells with a helper construct, together with the HIV-HSAΔE and HIV-EGFPΔE reporter vectors and a VSV-G-encoding plasmid. HOS cells were used primarily because they had previously been shown to be readily infectable by pseudotyped vectors (23, 49). To test for expression of the reporter genes, cells were collected 3 days after infection and processed for quantitative FACS analysis (Fig. 2). Depending on the multiplicity of infection used, up to 85% of the cells expressed HSA and up to 55% of the cells were EGFP positive (Fig. 2), indicating that the improved vector system was as efficient as our original two-component system in delivering the *HSA* reporter gene to HOS cells (49).

Effects of accessory proteins and IN on efficiency of the vector system. To test the influence of the various accessory proteins and IN on the efficiency of the gene transfer system,

virus stocks were generated by using a number of different helper constructs, together with the HIV-neoΔE or HIV-HSAΔE reporter vectors and a VSV-G-encoding plasmid. Virus stocks were harvested 60 h after transfection and subsequently used to infect HOS cells. Selection for growth in the presence of G418 allowed us to quantify the infection titer of each virus stock by counting G418-resistant colonies. The results presented in Table 1 show that pseudotype formation with the HIV-neoΔE vector was very efficient, with titers of the unconcentrated virus reaching 8.5×10^7 G418-resistant CFU/μg of p24. Colony formation was strictly dependent on the presence of a helper construct. In the absence of a helper construct, the titers were below the detection limit of the assay (Table 1). Also, a functional IN protein was necessary for efficient gene transfer. A helper construct with a defective IN core domain (C-Help IN) (15) yielded a G418 titer 3 orders of magnitude below the one obtained with helper constructs encoding a functional IN (Table 1), demonstrating that IN is required for efficient gene transfer to occur. To test the impact of the other accessory proteins on the formation of infectious pseudotypes, helper constructs lacking functional *vif*, *vpr*, and/or *vpr* coding regions were designed and tested. The results presented in Table 1 show that helper constructs with a mutated *vpr* coding region, carrying either a frameshift mutation at position 5743

(C-Help *vpr*⁻) or a 115-bp deletion (C-Help Δ *vpr*), yielded vector particles that efficiently transduced HOS cells. In addition, C-Help constructs lacking the Vif, Vpr, and Vpu accessory proteins (C-Help Δ vif Δ vpr Δ vpu) produced pseudotypes that efficiently delivered the *neo* reporter gene into HOS cells. We used quantitative FACS analysis to test in parallel the functionality and efficiency of the various helper constructs by monitoring the expression of the *HSA* reporter gene. HOS cells were stained 3 days after infection for cell surface-expressed HSA with fluorescein isothiocyanate-labeled anti-HSA antibody. No signal above background levels was seen with cells that had been infected with HIV-HSA Δ E stocks previously prepared by using an IN-deficient helper construct (C-Help IN) (Table 1) or in the presence of 10 μ M zidovudine (AZT) (36). The C-Help *vpr*⁻, C-Help Δ *vpr*, and C-Help Δ vif Δ vpr Δ vpu constructs produced pseudotypes that infected HOS cells as efficiently as virus stocks that had been prepared by using the C-Help construct producing intact Vif, Vpr, and Vpu, thus confirming the results obtained in assays using G418 selection. Taken together, these results indicate that Vif, Vpr, Vpu, and Nef are dispensable for infection of proliferating HOS cells.

The presence of a functional *tat* coding region in the vector enhances pseudotype titers. With a view toward constructing safe and efficient HIV-1-based gene transfer vectors, we designed constructs with deletions of various lengths affecting the *gag*, *pol*, *vif*, *vpr*, *vpu*, and 5' *tat* and *rev* coding regions and tested the efficiency of formation of G418-resistant colonies. To do this, the previously described HIV-neo vector (49) was modified. A schematic diagram of this vector and the various deletion derivatives is shown in Fig. 3. In the HIV-neo Δ E construct, sequences from the *Spe*I site (position 1506) up to the *Eco*RI site (position 5742) were deleted, thus completely eliminating the *pol* and *vif* genes and truncating the *gag* and *vpr* coding regions, but the 5' *tat* and *rev* exons remain intact. In contrast, HIV-neo Δ Tat (-) contains a mutated *tat* coding region carrying two consecutive stop codons after amino acid 10, leading to a truncated version of Tat (19). However, since the *rev* coding region is unaltered, functional Rev protein is produced from this vector. In the HIV-neo Δ S construct, the 5' *tat* and *rev* exons are retained but the splice acceptor site at position 5777 is missing; in plasmid HIV-neo Δ N, sequences up to the *Nde*I site at position 6399 were removed, thereby deleting the 5' *tat* and *rev* exons and 178 nucleotides of the gp160 coding region. The efficiency of each of these HIV-neo constructs was tested by using the C-Help construct to produce pseudotyped virus stocks and testing the viruses on HOS cells. The efficiency of formation of G418-resistant colonies differed markedly among the various HIV-neo deletion constructs. While the HIV-neo Δ E construct was as efficient as the original HIV-neo construct in generating G418-resistant colonies upon infection of HOS cells [$4.8 \pm 0.6 \times 10^7$ versus $3.5 \pm 0.7 \times 10^7$ CFU/ μ g of p24], the HIV-neo Δ Tat (-), HIV-neo Δ S, and HIV-neo Δ N derivatives gave six- to ninefold-reduced yields of G418-resistant colonies relative to that obtained with HIV-neo Δ E. The NL-neo vector was constructed according to the design of Parolin et al. (41) and Naldini et al. (38), with the expression cassette located 3' of the RRE. The titer obtained with this vector was comparable with the titers obtained with the other vector constructs lacking Tat. This finding suggests that the presence of Tat in the vector has a marked influence on the efficiency of formation of G418-resistant colonies.

Pseudotype formation using alternative Env glycoproteins. The capacity of HIV-1-based vectors to form pseudotypes with other Env proteins was investigated. The rabies virus G glycoprotein and the G protein of a related rhabdovirus, Mokola

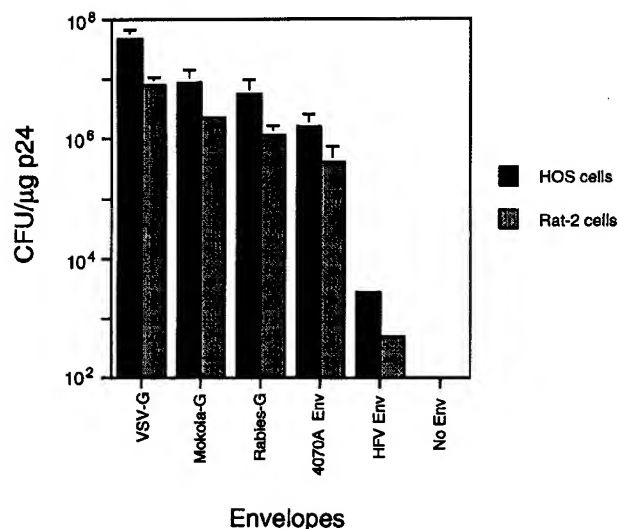


FIG. 4. Pseudotype formation using alternative Env proteins. HOS cells or Rat-2 cells were infected with HIV-neo Δ E pseudotypes carrying different Env proteins. Cells were trypsinized 3 days after infection and serially diluted into DMEM-FBS containing G418 (0.35 mg of active drug per ml). Colonies were stained 10 to 14 days later. 4070A Env, amphotropic MoMLV Env. Error bars represent standard deviations.

virus (*Lyssavirus* serotype 3), were tested, together with the Env protein of HFV and the amphotropic MoMLV 4070A Env, for the ability to yield infectious particles. All *env* coding regions were expressed under the control of the HIV-1 LTR. The normalized efficiency of formation of resistant colonies following packaging of the HIV-neo Δ E construct and subsequent infection of HOS cells and Rat-2 cells was determined. Figure 4 shows that VSV-G yielded up to 5×10^7 CFU/ μ g of p24 on HOS cells and up to 8×10^6 CFU/ μ g of p24 on Rat-2 cells. Particles pseudotyped with the Mokola virus and rabies virus G glycoproteins, and the MoMLV 4070A Env, yielded infectious titers of up to 9×10^6 CFU/ μ g of p24 on HOS cells and up to 2×10^6 CFU/ μ g of p24 on Rat-2 cells. The HFV Env also led to formation of HIV-1 pseudotypes, but the titers obtained (2.8×10^3 CFU/ μ g of p24 on HOS cells and 2.5×10^2 CFU/ μ g of p24 on Rat-2 cells) were 4 orders of magnitude below those obtained with VSV-G. These results underscore the flexibility of the HIV-1 vector system to form infectious pseudotypes, but they show its limitation as far as the HFV Env is concerned.

Assay for the generation of replication-competent virus. We next wished to determine if replication-competent virus that would subsequently be able to replicate in human T cells was produced during transient transfection. Two parallel cultures of the human H9 T-cell line (31) were infected with an HIV-neo Δ E stock pseudotyped with VSV-G for 3 days and subsequently split 1:4. This procedure was repeated five more times over a period of 35 days, and the supernatants were assayed for the presence of p24. The data in Fig. 5A show that extracellular p24 levels decreased with increasing numbers of transfer and that final p24 concentrations reached background levels. A similar decrease in p24 levels was observed in cultures infected with HIV-neo Δ E stocks harboring the HIV-1 HXB2 Env protein (Fig. 5B). This finding indicates that there was no substantial de novo production of p24-positive particles within the detection limits of the assay. However, cultures infected with HIV-neo stocks harboring the HIV-1 Env protein yielded ex-

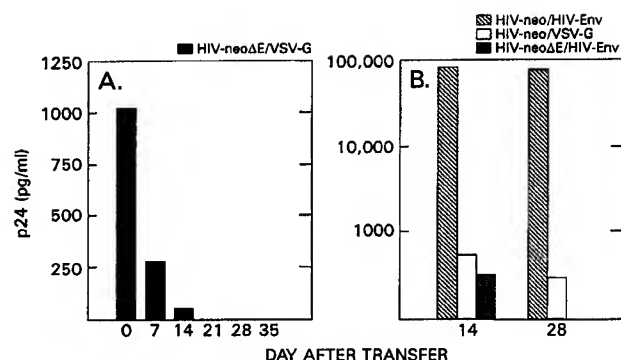


FIG. 5. Detection of replication-competent virus. (A) Duplicate cultures of human H9 cells (0.9×10^6 to 1.2×10^6 cells per culture) were infected with 0.5 ml of HIV-neoΔE pseudotype stock. The cells were split six times at a ratio of 1:4 over a period of 35 days; p24 was assayed throughout the experiment. Average p24 levels are shown. (B) H9 cells were infected with HIV-neo and HIV-neoΔE stocks containing the HIV-1 Env protein (HIV-neo/HIV-Env and HIV-neoΔE/HIV-Env, respectively). HIV-neo stocks pseudotyped with VSV-G (HIV-neo/VSV-G) were run in parallel. The cells were split once a week, and p24 concentrations were determined. The p24 values observed at days 14 and 28 are shown.

tracellular p24 levels up to 80 ng/ml, and these levels remained high after five transfers (Fig. 5B). This finding is consistent with the view that replication-competent virus was emerging. HIV-neo vectors pseudotyped with VSV-G yielded p24 levels

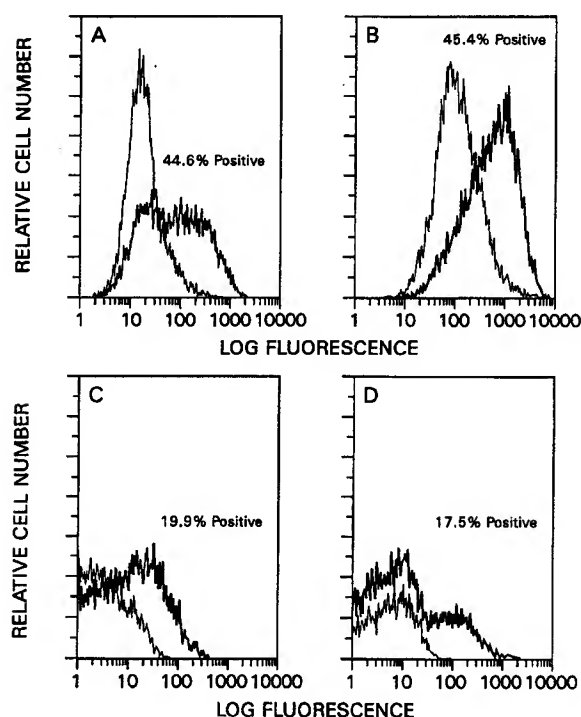


FIG. 6. Relative infection efficiencies of dividing and nondividing cells. Non-irradiated (A) and irradiated (B) HOS cells were infected with pseudotyped HIV-HSAΔE vector stocks and subjected to FACS analysis 3 days later. Approximately 5×10^5 cells were plated into six-well plates and infected with 0.25 ml of virus stock. Dividing (C) and contact-inhibited (nondividing) (D) HSFs were infected with pseudotyped HIV-EGFPΔE vector stocks and subjected to FACS analysis 3 days after infection. Between 0.5×10^5 and 2.5×10^5 cells in six-well plates were infected with 0.25 ml of virus. HIV-neoΔE virus stocks were used as mock controls. Thick lines, HIV-HSAΔE- and HIV-EGFPΔE-infected cells; thin lines, mock-infected cells.

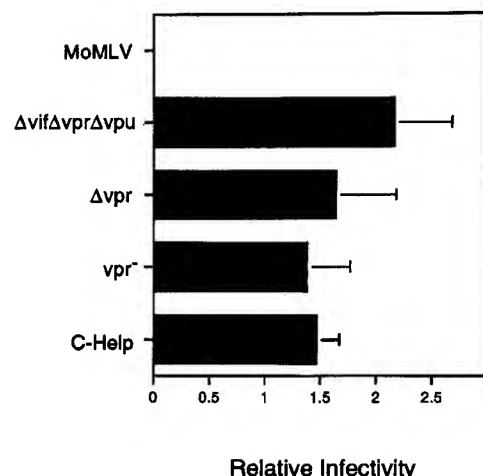


FIG. 7. Infection of growth-arrested HOS cells. Irradiated (4,000 rads) or nonirradiated HOS cells were infected with HIV-HSAΔE vector stocks previously prepared by using different helper constructs and subjected to FACS analysis 3 days later. Approximately 5×10^5 cells in six-well plates were infected with 0.25 ml of VSV-G-pseudotyped virus stocks. The relative infectivity represents the percentage of nondividing (irradiated) HSA-positive cells versus the percentage of dividing (nonirradiated) HSA-positive cells. The various helper constructs and the MoMLV-derived G1-HSA control vector stock are indicated. Error bars represent standard deviations.

around 500 pg/ml after the third transfer and around 300 pg/ml after the fifth. Low but constant p24 levels were expected in this case, as the HIV-neo vector backbone contains a functional p24 coding region.

Transduction of nondividing cells. The ability of the newly designed HIV-1 vector system to mediate gene transfer into nondividing cells was analyzed by transducing growth-arrested HOS cells, contact-inhibited primary HSFs, postmitotic rat cardiac myocytes, and postmitotic rat cerebellar neurons. The relative efficiency of gene transfer into dividing and nondividing cells was investigated first. G_2 -arrested HOS cells were prepared by γ irradiation (26). Cell cycle analysis of the irradiated cells showed that up to 80% of the cells were in G_2 (36). Such cells were subsequently infected with HIV-HSAΔE pseudotype stocks and analyzed by quantitative FACS analysis 3 days later. The numbers of HSA-positive cells were similar for irradiated (44.6% [Fig. 6B]) and nonirradiated (45.5% [Fig. 6A]) cells. Primary HSFs were growth arrested by being allowed to reach contact inhibition upon cultivation in medium containing 10% FBS for 3 weeks. Such cells have previously been shown to be highly enriched for populations in G_0 and/or G_1 (49, 61). Dividing control HSFs were prepared by subcultivation 2 days before infection. Dividing and nondividing HSFs were infected with HIV-EGFPΔE pseudotype stocks and analyzed by quantitative FACS analysis 3 days later. The fraction of EGFP-positive, dividing HSFs was 19.9% (Fig. 6C), while 17.5% of the contact-inhibited, nondividing HSFs (Fig. 6D) were EGFP positive, indicating that infection efficiency was independent of the proliferative status of the cells. Given that only about 20% of the infected HSFs were EGFP positive, a control infection was done with HOS cells in parallel. The results revealed that over 60% of these cells were EGFP positive, indicating that there are quantitative differences in the abilities of VSV-G-pseudotyped HIV-1-based vectors to infect primary cells versus established cell lines.

The influence of the Vpr, Vif, and Vpu accessory proteins on the efficiency of reporter gene transfer into G_2 -arrested HOS

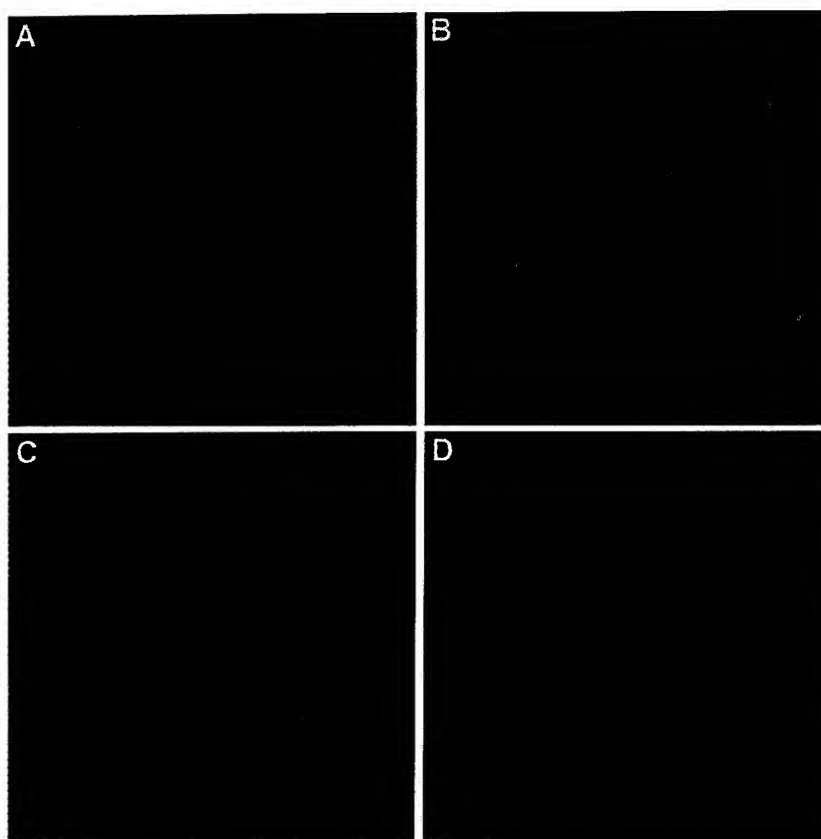


FIG. 8. Infection of contact-inhibited HSFs. (A to C) HSFs infected with HIV-EGFPΔE pseudotypes. (A) *vif*, *vpr*, and *vpu* genes present in helper construct; (B) all accessory protein-encoding genes absent in helper construct; (C) IN control; (D) mock infection.

cells and contact-inhibited HSFs was determined next. HOS cells were irradiated (4,000 rads), subsequently infected with HIV-HSAΔE vector stocks, and processed for quantitative FACS analysis 3 days later. Subconfluent HOS cells were similarly infected and processed in parallel, and the ratios of the percentages of HSA-positive, nondividing (irradiated) HOS cells versus HSA-positive, dividing HOS cells were determined. These ratios varied only slightly, regardless of the C-Help construct used (Fig. 7). The MoMLV-derived G1-HSA vector stock served as a control. It did not transduce G₂-arrested cells above background levels, indicating that the G₂ block was effective. Contact-inhibited, nondividing HSFs were infected using HIV-EGFPΔE vector stocks and inspected by fluorescence microscopy 29 days later (Fig. 8). Vector stocks assembled from packaging constructs in which all accessory protein-encoding regions were absent were as efficient as the corresponding stocks assembled from wild-type constructs (compare Fig. 8B and A). A vector stock assembled from an IN-deficient helper was severely impaired (Fig. 8C). Taken together, these results show that the *vif*, *vpr*, and *vpu* genes in the helper construct are dispensable for infection of growth-arrested HOS cells and contact-inhibited HSFs.

Primary neonatal rat ventricular myocytes prepared from the hearts of 2- to 3-day-old rats were prepared and infected 5 days later with HIV-EGFPΔE and HIV-ShlacZΔE pseudotypes or a pseudotyped MoMLV-lacZ control vector. The results presented in Fig. 9A to C show that myocytes had been successfully infected in that the EGFP-positive cells clearly overlapped the rhodamine-positive cells, labeled with a myosin-

specific antibody; Fig. D to F show that none of the accessory proteins are required to generate virus that can infect such myocytes, as judged from the X-Gal staining; Fig. 9H (IN control) shows that the signals observed are not due to pseudotransduction. Moreover, the cells were no longer dividing at the time of infection, as judged from results for the MoMLV-lacZ virus control (Fig. 9G).

Cerebellar granule cells, also nondividing cells (60), were prepared from 8-day-old rat pups and infected 1 day later with HIV-ShlacZΔE and HIV-ShlacZΔN stocks pseudotyped with VSV-G. The HIV-ShlacZΔN vector is similar to the HIV-neoΔN vector because it lacks the 5' *tat* and *rev* exons. Expression of the reporter gene was detected by β-galactosidase staining using the X-Gal substrate 3 days later. Up to 30% of the granule cells were X-Gal positive after overnight incubation (Fig. 10A and B). HIV-ShlacZΔE stocks previously prepared by using a *vpr*-deficient helper construct or helper constructs lacking all accessory proteins were as efficient as the corresponding stocks that had been prepared by using wild-type helper constructs (Fig. 10C and D). There was no X-Gal staining in granule cells that were infected in the presence of 10 μM AZT (Fig. 10F), suggesting that the staining is specific. MoMLV-based vectors encoding β-galactosidase did not produce signals above background levels (36).

DISCUSSION

We have developed an efficient three-component packaging system to produce HIV-1 pseudotypes. The design of the sys-

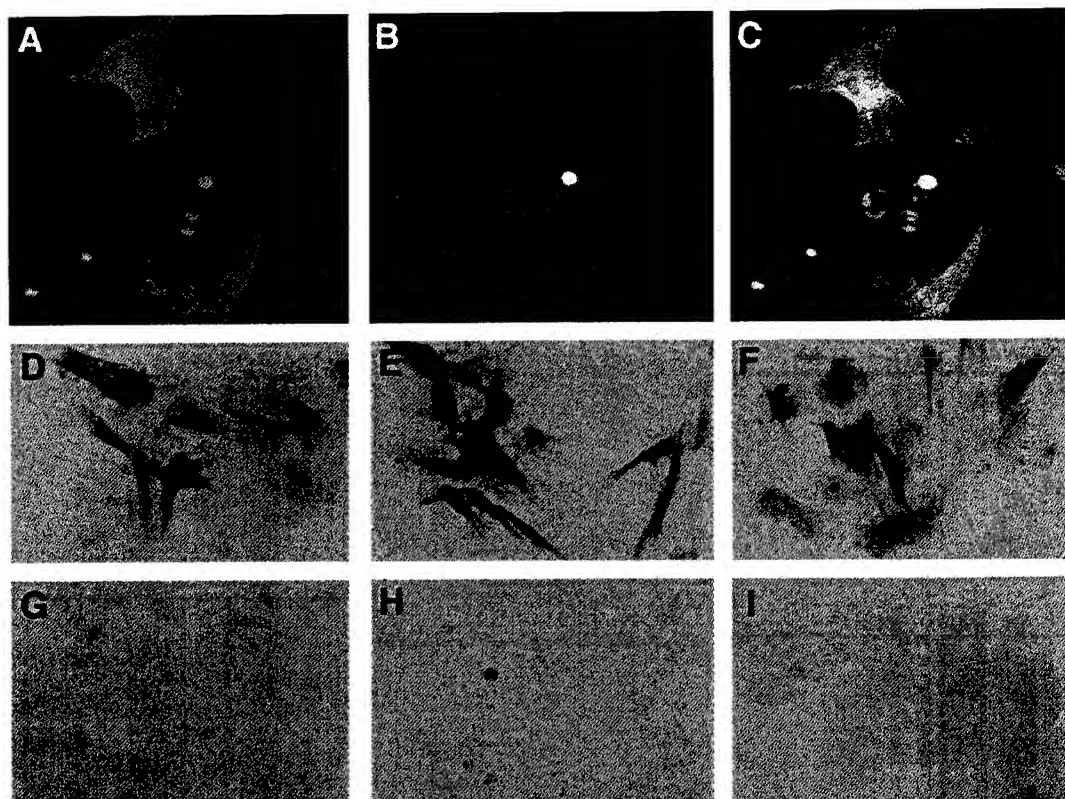


FIG. 9. Infection of postmitotic rat cardiac myocytes. (A to C) Rat cardiac myocytes infected with HIV-EGFP Δ E pseudotypes. (A) EGFP fluorescence; (B) myosin-specific rhodamine fluorescence; (C) combination of panels A and B. (D to I) Cardiac myocytes infected with HIV-ShlacZ Δ E. (D) *vif*, *vpr*, and *vpu* genes present in helper construct; (E) *vif* and *vpu* genes present in helper construct; (F) all accessory protein-encoding genes absent in helper construct; (G) MoMLV-lacZ vector; (H) IN control; (I) mock infection. Approximately 2×10^5 myocytes in six-well plates were infected with 0.3 to 0.5 ml of the various virus stocks. The cells were stained with X-Gal or processed for immunofluorescence 3 days after infection.

tem is based on the concept of split packaging systems that have been available for oncoretroviruses for over a decade (34). Transient three-component packaging systems that include the native HIV-1 gp160 have been available for some time (5, 45, 53, 50, 59), but their efficiency was generally quite low, with titers on the order of 10^3 to 10^4 transducing units per ml of supernatant. Naldini et al. (38) were the first to describe an HIV-1-based three-component system that involves heterologous Env proteins. Their pseudotype titers with VSV-G or the MoMLV amphotropic Env protein ranged from 1×10^5 to 4×10^5 transducing units per ml, based on β -galactosidase staining of transduced cells. The efficiency of our three-component system appears to be higher. The titers obtained with *neo* vectors were between 1×10^6 and 2×10^7 CFU/ml, depending on the vector construct used. Titers above 10^7 CFU/ml (up to 8×10^7 CFU/ μ g of p24) were routinely obtained provided that a functional Tat coding region was present in the vector. Vector constructs lacking a functional Tat protein typically yielded titers of around 10^6 CFU/ml (4×10^6 to 8×10^6 CFU/ μ g of p24).

The use of heterologous Env proteins such as VSV-G is assumed to preclude the formation of replication-competent HIV-1, and the separation of the helper functions from the vector functions further adds to the safety of the system. However, there is still substantial sequence overlap between the vector and helper constructs as far as *gag* sequences and sequences spanning the RRE are concerned. Theoretically, these regions of overlap could lead to recombinants that could re-

constitute packageable helper constructs, and eliminating them will be mandatory in the long run to make safe HIV-1 vectors. Rev-independent HIV-1 helper constructs as described by Schneider et al. (52) and Srinivasakumar et al. (57) may be helpful in this respect. They may facilitate the design of packaging systems that lack RRE altogether, thus reducing the likelihood of recombination.

We have previously reported that functional *vpr* and *nef* coding regions are not required for generating high-titer HIV-1 vector stocks and for the subsequent transduction of proliferating and growth-arrested cells (49). The results presented here extend these findings and are consistent with the view that the Vif, Vpr, Vpu, and Nef functions are not required for the efficient transduction of proliferating and growth-arrested HOS cells in vitro. Also, the efficiency of transduction of postmitotic rat cardiac myocytes and cerebellar neurons was the same regardless of whether functional Vif, Vpr, or Vpu was present in the packaging construct. Some or all of the HIV-1 accessory proteins may be needed in other cell types to achieve efficient transduction. Zufferey et al. (65), Kafri et al. (20), and Kim et al. (21) recently described HIV-1 helper constructs in which several of the genes encoding accessory proteins have been deleted. Vector constructs packaged with these multiply deleted helper constructs retained the ability to transduce growth-arrested cells and monocyte-derived macrophages in culture and could efficiently deliver genes in vivo into muscle and adult neurons. Vpr and Vif were found to be required for efficient gene delivery into the liver (20).

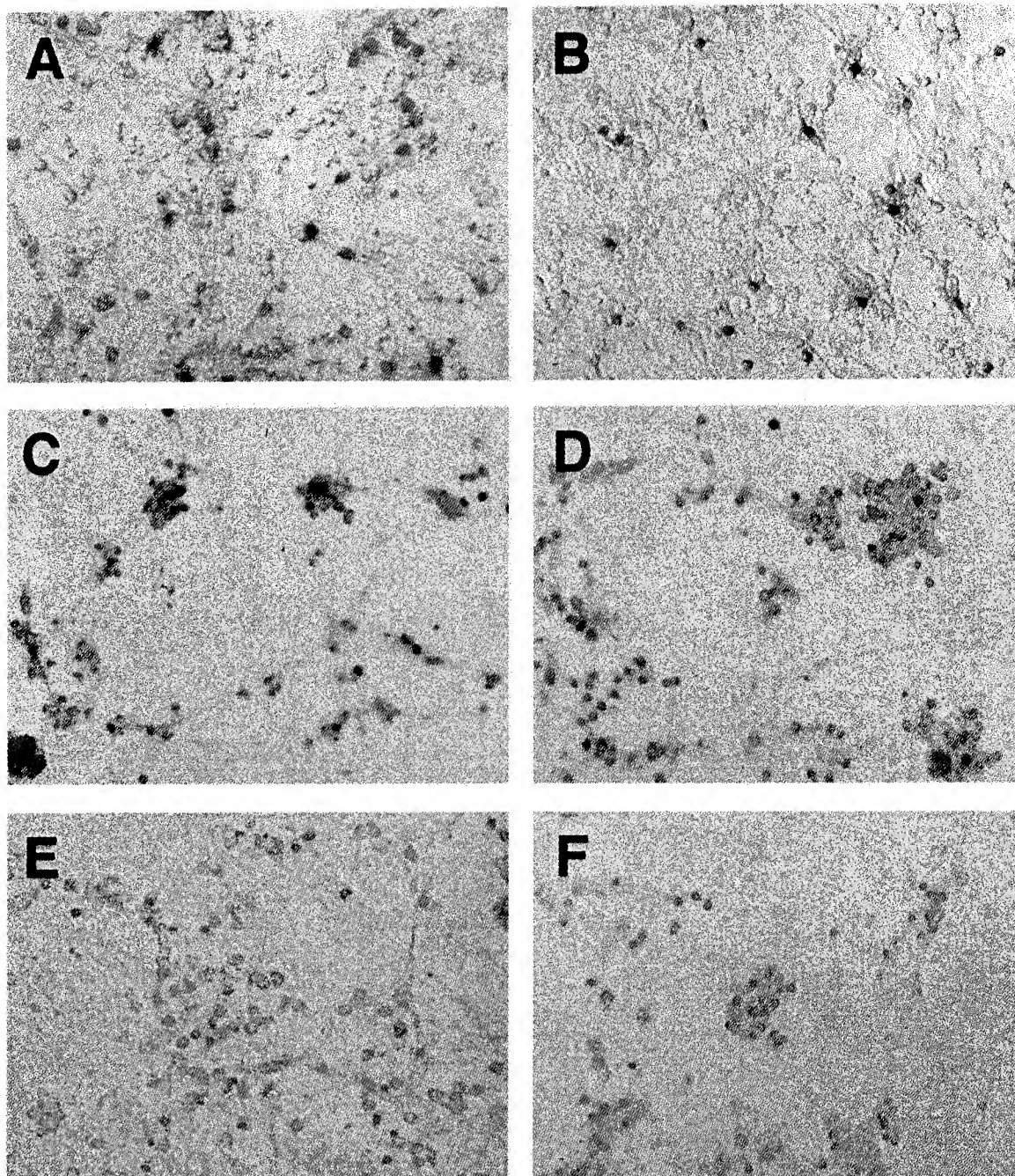


FIG. 10. Infection of postmitotic rat cerebellar granule cells. (A, C, D, and F) Rat cerebellar granule cells infected using HIV-ShlacZΔE pseudotypes; (B) rat cerebellar granule cells infected using HIV-ShlacZΔN pseudotypes; (C) *vpr* gene absent in helper construct; (D) all accessory protein-encoding genes absent in helper construct; (E) mock infection; (F) cells infected in the presence of 10 μ M AZT. Approximately 1.6×10^5 granule cells in 35-mm-diameter dishes were infected with 0.3 ml of virus stock and stained with X-Gal 3 days later.

In the study presented here, we compared different HIV-neo vector constructs for the ability to yield G418-resistant colonies. The HIV-neoΔE vector harbors intact 5' *tat* and *rev* exons, while the HIV-neoΔTat (–) vector harbors a point-mutated 5' *tat* exon and lacks the capacity to produce functional Tat. In the HIV-neoΔN construct, the 5' *tat* and *rev* exons are missing. Compared to the HIV-neoΔTat (–) and HIV-neoΔN constructs, the HIV-neoΔE construct exhibited

an approximately 6- to 10-fold-higher yield of G418-resistant colonies. This is probably due to the production of functional Tat by the HIV-neoΔE vector in infected target cells and concomitant improved *neo* gene expression from the Tat-regulated viral LTR, thus boosting the yield of G418-resistant colonies. This assumption is supported by the findings of Parolin et al. (42), who showed that Tat-driven reporter gene expression was substantially higher than expression of vectors

that used heterologous internal promoter elements; the difference in expression was not due to improved packaging of the vector constructs. Although Tat-driven expression is very robust, the continuous production of Tat and Rev in the target cell may be detrimental, as Tat has been implicated in a number of effects besides its contribution to transcription, including cytotoxicity (8). However, for certain *in vitro* experiments, such as screening of cDNA expression libraries, the high efficiency of gene transfer may be advantageous and the presence of Tat in the vector construct may be desirable.

HIV-1 appears to be flexible in terms of forming pseudotypes, thereby allowing the expansion of its host range. In addition to VSV-G and the MoMLV amphotropic Env, rabies virus and Mokola virus G proteins formed pseudotypes, although the yield of G418-resistant colonies was highest with VSV-G pseudotypes. This difference may reflect, in part, receptor levels, pseudotype stability, and efficiency of pseudotype formation. Poor pseudotype formation involving the HFV Env may explain the low yield of G418-resistant colonies. This possibility is supported by the recent findings that the HFV Env cytoplasmic domain harbors an endoplasmic reticulum retention signal (18, 28), which may affect the proper assembly of HIV-1 pseudotypes at the plasma membrane.

The *lacZ* reporter gene can lead to pseudotransduction artifacts under conditions where highly concentrated VSV-G-pseudotyped MoMLV-derived vectors are used (29). We have ruled out pseudotransduction by including an IN control and by using AZT during infection. Also, because our titers are high, there is generally no need to concentrate the virus.

The three-component HIV-1 packaging system described here is efficient, robust, and safe. However, the design of efficient packaging cell lines is mandatory. The various constructs described here will be helpful in constructing such cell lines.

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